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[1]Passed away on October 20, 2007
[2]Passed away on June 11, 2007
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Contents

Contents

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Contents

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Diet and epigenetics in colon cancer

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Abstract

Over the past few years, evidence has accumulated indicating that apart from genetic alterations, epigenetic alterations, through e.g. aberrant promoter methylation, play a major role in the initiation and progression of colorectal cancer (CRC). Even in the hereditary colon cancer syndromes, in which the susceptibility is inherited dominantly, cancer develops only as the result of the progressive accumulation of genetic and epigenetic alterations. Diet can both prevent and induce colon carcinogenesis, for instance, through epigenetic changes, which regulate the homeostasis of the intestinal mucosa. Food-derived compounds are constantly present in the intestine and may shift cellular balance toward harmful outcomes, such as increased susceptibility to mutations. There is strong evidence that a major component of cancer risk may involve epigenetic changes in normal cells that increase the probability of cancer after genetic mutation. The recognition of epigenetic changes as a driving force in colorectal neoplasia would open new areas of research in disease epidemiology, risk assessment, and treatment, especially in mutation carriers who already have an inherited predisposition to cancer.

Key words: Colon cancer; Diet; DNA methylation; Epigenetics; Nutrition

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INTRODUCTION

The incidence of colorectal cancer (CRC) varies up to 25-fold between countries. Highest rates are found in Westernized societies, such as the USA, Australia and New Zealand and lowest rates are found in Africa and India. Evidence that causes of CRC are largely environmental comes from studies where people who migrate from low- to high-risk areas of the world reach the incidence of cancer in a high-risk country even over one or two generations. In these migration studies the main characteristic has been a change from a prudent diet to a Westernized diet with higher intake of energy dense foods and lowered physical activity.

It has been speculated that epigenetic changes in the genome might explain these ecological findings. Epigenetics are related to the inheritance of information based on gene expression levels, as opposed to genetics, which refers to information transmitted on the basis of gene sequence. In recent years, evidence has accumulated indicating that apart from genetic changes, epigenetic alterations play a major role in the initiation and progression of CRC[1,2].

Different environmental conditions may confer different activity to the same genes. Epigenetic processes are essential in normal development and differentiation but may sometimes be misdirected and predispose to cancer. Epigenetic events, such as altered methylation patterns (hypermethylation and hypomethylation), post-translational modifications of histones, and chromatin remodeling, can lead to inactivation of tumor suppressor genes, activation of oncogenes, or altered imprinting patterns. The best-known epigenetic marker is DNA methylation, described to occur in complex chromatin networks and is influenced by the modifications in histone structure that are commonly disrupted in cancer cells[3,4]. Diet is a major aspect of the environment which may influence DNA methylation thus providing an important common link between cancer and nutrition[5].

COLORECTAL CANCER

CRC is the second most common cause of cancer-related deaths in the Western world although a worlwide population-based study has shown that 5-year relative survival for CRC seems to be generally higher in high-income countries[6]. Approximately 50% of the population in Western countries will develop adenomatous lesions of the colon, but only a minor proportion will develop cancer[7]. CRCs are mainly sporadic, and inherited factors have been estimated to be of importance in about 30% of all CRCs[8]. While many inherited predisposing factors are still unidentified, 13% of CRCs have been reported to occur in association with the two most common inherited colon cancer-predisposition syndromes, i.e. hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP), which are caused by germline mutations in DNA mismatch repair (MMR) genes and the adenomatous polyposis coli (*APC*) tumor suppressor gene, respectively[7,9]. Susceptibility to HNPCC and FAP is inherited in an autosomal dominant manner. At the cellular level, these genes act recessively, i.e. inactivation of the wild-type allele (loss-of-function) is required for an altered cell phenotype[10]. The lifetime risk of cancer for individuals carrying an inherited germline mutation in a *MMR* gene or *APC* is high, but cancer develops only as the result of the progressive accumulation of somatic genetic and epigenetic alterations in several other genes involved in various cellular pathways.

MAJOR PATHWAYS OF COLORECTAL CARCINOGENESIS

Analyses of tumors associated with FAP and HNPCC have helped to understand many details of the molecular pathogenesis of CRC in general[11]. The development of CRC is a multi-step process beginning with the transformation of normal colonic epithelium, first to benign adenomatous polyps and eventually to invasive carcinoma, and finally metastasis[7,12]. Mutational inactivation of *APC* plays a rate-limiting role in about 70% of sporadic CRCs[13]. Epigenetic silencing of *APC* through promoter hypermethylation has also been reported in a number of sporadic colorectal adenomas and carcinomas[14]. The principal tumor promoting character of inactivated *APC* is the insufficient degradation of β-catenin, a key mediator of the Wnt signaling pathway. Consequently, more β-catenin enters the nucleus and overactivates Wnt signaling, resulting in transcriptional activation of Wnt/TCF4 (T-cell factor 4) target genes (e.g. *c-myc* and *cyclin D1*), initiating transformation of intestinal epithelial cells[15,16]. Physiologically, the Wnt pathway is essential for the maintenance of intestinal crypt progenitor compartments[17]. Tumors associated with *APC* mutations are characterized by chromosomal instability (CIN)[11].

Another pathway of carcinogenesis involves the cellular DNA MMR system. Cells defective in MMR are characterized by microsatellite instability (MSI) phenotype. MMR deficiency results in activation of the mutator pathway which creates accumulating frameshift mutations in many growth-regulatory genes with coding microsatellites, thus promoting genome-wide genetic instability[18]. Many of these affected genes are general tumor suppressor genes, as well as genes that function in DNA mismatch repair, Wnt signaling, and apoptotic pathways. MMR deficiency thus promotes the activation of many pathways, which lead to the expression of genes that favor cell growth[11]. Compared to CIN, MSI is a feature of a smaller subset of cancers; it is a hallmark for HNPCC tumors and is reported in approximately 15%-25% of all CRCs and 10%-20% of all endometrial and gastric cancers[7,19]. In HNPCC, most tumors are due to germline mutations in the MMR genes *MLH1*, *MSH2,* and *MSH6* (http://www.insight-group.org/). However, most sporadic MSI tumors are associated with epigenetic silencing (hypermethylation) of the *MLH1* promoter[20,21].

EPIGENETICS IN COLON CANCER

Epigenetics is defined as heritable changes in gene expression that are not due to any alteration in the DNA sequence[22]. It has been proposed that heritable changes in gene activity due to DNA modification should be referred to as epimutations to distinguish them from classical gene mutations. As DNA methylation is known to be essential for the normal control of gene activity during development, defects in methylation may have severe phenotypic consequences. Recently, considerable attention has been focused on the role of CpG islands (CGI) hypermethylation in the molecular pathogenesis of CRC. These islands are usually not methylated in normal cells[23,24]. The finding of aberrant *MLH1* promoter hypermethylation in sporadic MSI CRCs dramatically illustrated the role of epigenetic changes as potential pathogenetic alterations in cancer. Furthermore, in cell lines, reversion of the methylation using demethylating agents frequently restores expression of *MLH1,* demonstrating that methylation in fact induces gene silencing. These data strongly suggested that such aberrant *MLH1* promoter methylation is a cause of colon carcinogenesis[20]. The role of aberrant CGI hypermethylation in colon carcinogenesis was later demonstrated in animal studies, where overexpression of de novo DNA methyltransferase DNMT3b accelerated tumor formation[25], whereas chronic administration of an oral inhibitor of DNA methylation dramatically reduced tumor formation in the mucosa[26].

To date, several hypermethylated genes are associated with colorectal neoplasia, including tumor suppressor, DNA repair, and cell cycle regulatory genes (e.g. *APC, CDH13, CHFR, MLH1, BRCA1, p14, p16, RARB2, SFRP1, WRN, RASSF1A, MGMT,* and *TIMP3*)[2,27]. These genes are being explored as biomarkers in clinical use for preventive and therapeutic interventions. Of

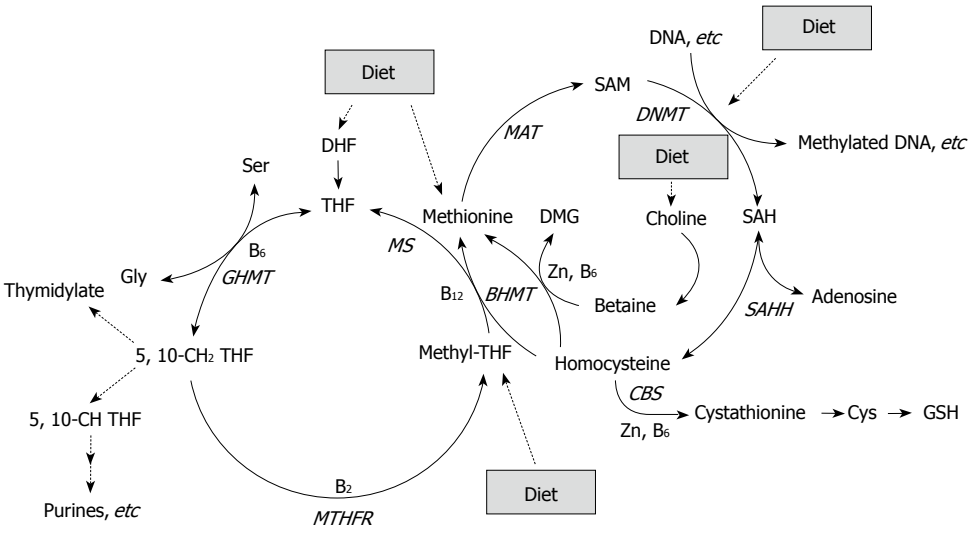

Figure 1 Folate, in the form of methyltetrahydrofolate (methyl-THF), is involved in remethylation of homocysteine to methionine, which is a precursor of SAM, the primary methyl group donor for most biological methylation reactions, also in DNA[64]. Folate deficiency may thus enhance CRC through an induction of genomic DNA hypomethylation. Expression of several enzymes (GHMT, MTHFR, BHMT, MAT, SAHH, CBS) involved in methyl metabolism can be regulated by diet such as availability of nutrients including essential amino acids, vitamins B2, B6 and B12, and Zinc (Zn).

these, promoter hypermethylation of *p16*^{INK4A}, *MGMT*, and *MLH1* have been suggested to be useful markers for risk assessment and hypermethylation of *APC* in the detection of colorectal carcinoma[27,28]. Hypermethylation of *MLH1* may also serve as a second "hit" inactivating the wild type copy of the gene in HNPCC-associated tumorigenesis[29].

Hypermethylation occurs at different cancer stages and can be associated with either of the two major pathways of colorectal carcinogenesis. For a panel of genes, the expression profiles measured in histologically normal mucosa have been reported to differ significantly between patients with and without colorectal cancers[30]. Moreover, different epigenetic phenotypes have been found to distinguish the colonic mucosa in individuals who develop sporadic MSI-positive and MSI-negative colorectal tumors[31]. These methylation phenotypes may underlie different developmental pathways that occur in these tumors. Recently, inactivation of tumor suppressor genes by promoter methylation was further shown to follow patterns characteristic of tumor type (CRC versus endometrial carcinomas) and family category (familial CRC *versus* sporadic) and was strongly influenced by *MLH1* promoter methylation status in all categories[32]. A phenomenon called CpG island methylator phenotype (CIMP) has been described in a subgroup of colorectal adenomas and carcinomas[33,34]. In CIMP tumors, multiple tumor suppressor genes are inactivated by promoter hypermethylation[35], and CIMP has been suggested to provide an alternative pathway to promote colon cancer resembling in many features MSI tumors, although they are microsatellite stable[36,37].

DIET AND COLON CANCER

Colorectal cancer is a disease associated with increasing age and there is strong evidence that the risk of CRC can be modified by lifestyle and environmental factors[38,39]. It has been demonstrated that diet may account for or prevent as much as 80% of CRC incidence[40]. Diet may affect gut mucosa either directly from the luminal side or indirectly through whole-body metabolism. Food-derived compounds that are constantly present in the intestine, or the blood content of nutrients, hormones and growth factors, may shift cellular balance toward harmful outcomes, such as increased susceptibility for genetic and epigenetic changes in a genome.

There is a strong assumption that diet, especially Western-type diet, contributes to the development of CRC. In 2007, the World Cancer Research Fund and the American Institute for Cancer Research published their 2nd comprehensive review entitled 'Food, Nutrition, Physical Activity and the Prevention of Cancer; a Global Perspective' (http://www.dietandcancerreport.org) supporting this belief. Based on mainly prospective cohort studies it was concluded that there is convincing evidence that red and processed meat, substantial consumption of alcoholic drinks, body fat and abdominal fatness, and the factors that lead to greater adult attained height or its consequences are causes of CRC. In addition, foods containing dietary fiber, garlic, milk and calcium probably protect against this cancer. Moreover, non-starchy vegetables, fruits, fish, foods containing folate, vitamin D, or selenium may protect against CRC, and foods containing animal fats or sugar may cause CRC. In a recent study, CRC re-occurrence was also shown to be significantly higher in subjects consuming the most Westernized diet compared to diets with more fiber and less fat and sugar[41].

The complex interactions of dietary components with each other and with metabolism make it difficult for epidemiological methods to specifically identify the components which might induce or prevent CRC (Figure 1). Murine models such as *Min*/+ mice, which

is the best-characterized mouse colonic neoplasia model and analogous to the human FAP syndrome[42] have provided a valuable tool, allowing thorough dissection of the effects of specifically controlled diets. A comprehensive list of compounds that have been tested for CRC promotion or prevention in this animal model can be found on Corpets website at http://www.inra.fr/reseau-nacre/sci-memb/corpet/Data/table.php?file=Min-mice.txt. Min (multiple intestinal neoplasia) is an autosomal dominant trait involving a nonsense mutation in codon 850 of the murine *APC* gene. As in humans, the mutation predisposes to intestinal tumorigenesis. In both *Min*/+ mice and healthy rats, different types of diet have been shown to cause considerable changes in intestinal cell signalling pathways (PKC, NF-κB, β-catenin and COX-2, cyclin D1, E-cadherin, and p53) both in tumor tissue and also in the surrounding mucosa[43-46]. In particular, red meat[47] and a Western-type diet with low levels of calcium and cholecalciferol and high levels of n-6 polyunsaturated fatty acids[48,49] were shown to have unfavorable effects on tumor formation. These results are in line with the epidemiological evidence on the effect of red meat on CRC. Inadequate dietary folate has been shown to impair DNA excision repair in the rat colon in the absence of any chemical carcinogen, and increased folate supplementation inhibited intestinal polyp formation in *Min*/+ mice[50,51]. Moreover, wild-type mice have been shown to develop colon adenomas and an early invasive carcinoma in long-term diet experiments with a Western-type diet containing reduced calcium, vitamin D, folic acid and increased fat content, but without carcinogen exposure[52].

Experimental work has indeed shown that due to the heterozygous nature of fiber or fiber-rich foods, it is very difficult to draw firm conclusions on the effects of fiber on CRC. It is evident that different fiber types (soluble *vs* insoluble) and sources (grain, vegetables and fruits) may act in totally opposite ways in CRC[53]. In addition, fibers and fatty acids in the diet interact with each other and affect outcome[54]. The same is probably true with fiber and red meat, since odds ratios for CRC were substantially reduced in subjects who had a high level of red meat in their diet but who also consumed high levels of fiber when compared to subjects with low fiber intake[55]. Phenolic compounds from fruits and berries[56,57], curcumin from tumeric[58,59], epicallocatechin from green tea[60], and n-3 fatty acids from fish[61] have been widely studied as possible chemopreventive agents and have been shown to regulate different cell signaling pathways[62]. Moreover, the effect of polyphenols on DNA methylation is under active investigation[63-65].

DIET AND EPIGENETICS

The elucidation of the effects of diets on epigenetic changes in the intestinal mucosa is of great importance, as aberrantly methylated genes may have the potential to be early-detection and prognostic markers for colon cancer. Unlike genetic changes in cancer, epigenetic changes, such as alterations in methylation, are potentially reversible and, therefore, provide promising targets for preventive and therapeutic interventions. Diet is a major aspect of the environment that may influence DNA methylation, and studies on the role of specific foods, diet-derived compounds and different types of dietary patterns on cellular mechanisms and epigenetics in CRC are increasing. Especially interesting are nutrients, which are needed for nucleic acid and DNA synthesis and for the enzymes regulating their syntheses, e.g. essential amino acids, zinc, folate, and vitamins B-6 and B-12[66] (Figure 1). The most studied nutrient in this area is folate, and the portfolio of evidence from animal, human, and *in vitro* studies suggest that the effects of folate deficiency and supplementation on DNA methylation are gene- and site-specific, and appear to depend on cell type, target organ, stage of transformation, and the degree and duration of folate depletion[67].

As in the classical experiment of agouti mice, in which maternal diet, high in folates, choline and vitamin B-12 shifted the coat color of the offspring[68,69], diet may also induce epimutations detectable in the phenotype later in life in humans. Monozygotic twins have been shown to be epigenetically indistinguishable during the early years of life, while older monozygotic twins exhibited remarkable differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation[70]. Using the obesity-discordant monozygotic twins, Pietiläinen *et al*[71] have shown several changes in the transcription profiles of adipose tissue between the twins. The results showed the effects of acquired human obesity, which is independent of genetic factors, but may be related to epigenetic modulation of the genome.

A new and fascinating area in "diet and cancer" studies is the so called "fetal programming". In 1989, Barker *et al*[72] reported on an inverse relationship between birth weight and later glucose intolerance, hypertension, and hyperlipidemia and finally ischemic heart disease mortality in men born in England in the early 1900's. The hypothesis behind this relationship was that genes were epigenetically programmed in a way which favored energy storage in an energy poor environment and thus, later in an 'obesinogenic' or 'Western-type' environment, these same genes would lead to chronic diseases[73]. Genetic and early life environmental factors, even before birth, have also been shown to be important in adult height determination. Moreover, it has been suggested that the 'fetal programming' hypothesis or factors that promote linear growth in childhood might explain the epidemiological evidence on the clear dose-response relationship between greater adult height and a risk for CRC (http://www.dietandcancerreport.org). As has indeed been indicated by an animal model[74,75], the underlying mechanisms might include epigenetic modulation of growth hormone, insulin-like growth factors and sex hormone binding protein expression, all of which have an impact on height and growth and can be modulated by dietary means.

CONCLUSION

Epigenetic changes, such as alterations in methylation, may occur in normal cells, but may prime the mucosa for cancer progression. However, unlike genetic changes in cancer, these epigenetic changes are potentially reversible. The elucidation of the effects of food-derived compounds on epigenetic changes in intestinal mucosa is thus of great importance and will provide promising targets for preventive and therapeutic interventions. The identification of "methylation biomarkers" that are specific for colorectal tumorigenesis would be useful for risk assessment, especially in individuals who have an inherited susceptibility for CRC. Furthermore, those biomarkers required for the malignant phenotype would identify pathways important as therapeutic targets. In summary, the recognition of epigenetic changes as a driving force in colorectal neoplasia opens new areas of research in disease epidemiology, risk assessment, prevention, and treatment.

REFERENCES

1 **Feinberg AP**, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006; **7**: 21-33
2 **Wong JJ**, Hawkins NJ, Ward RL. Colorectal cancer: a model for epigenetic tumorigenesis. *Gut* 2007; **56**: 140-148
3 **Bernstein BE**, Meissner A, Lander ES. The mammalian epigenome. *Cell* 2007; **128**: 669-681
4 **Kouzarides T**. Chromatin modifications and their function. *Cell* 2007; **128**: 693-705
5 **Liu L**, Wylie RC, Andrews LG, Tollefsbol TO. Aging, cancer and nutrition: the DNA methylation connection. *Mech Ageing Dev* 2003; **124**: 989-998
6 **Coleman MP**, Quaresma M, Berrino F, Lutz JM, De Angelis R, Capocaccia R, Baili P, Rachet B, Gatta G, Hakulinen T, Micheli A, Sant M, Weir HK, Elwood JM, Tsukuma H, Koifman S, E Silva GA, Francisci S, Santaquilani M, Verdecchia A, Storm HH, Young JL. Cancer survival in five continents: a worldwide population-based study (CONCORD). *Lancet Oncol* 2008; **9**: 730-756
7 **Kinzler KW**, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159-170
8 **Lichtenstein P**, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med* 2000; **343**: 78-85
9 **de la Chapelle A**. The incidence of Lynch syndrome. *Fam Cancer* 2005; **4**: 233-237
10 **Vogelstein B**, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004; **10**: 789-799
11 **Narayan S**, Roy D. Role of APC and DNA mismatch repair genes in the development of colorectal cancers. *Mol Cancer* 2003; **2**: 41
12 **Markowitz SD**. Genetic and epigenetic alterations in colon cancer. *Annu Rev Genomics Hum Genet* 2002; **3**: 101-128
13 **Miyaki M**, Konishi M, Kikuchi-Yanoshita R, Enomoto M, Igari T, Tanaka K, Muraoka M, Takahashi H, Amada Y, Fukayama M. Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. *Cancer Res* 1994; **54**: 3011-3020
14 **Esteller M**, Sparks A, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Gonzalez S, Tarafa G, Sidransky D, Meltzer SJ, Baylin SB, Herman JG. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer Res* 2000; **60**: 4366-4371
15 **Morin PJ**, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 1997; **275**: 1787-1790
16 **Clevers H**. Wnt/beta-catenin signaling in development and disease. *Cell* 2006; **127**: 469-480
17 **Korinek V**, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, Clevers H. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet* 1998; **19**: 379-383
18 **Aaltonen LA**, Peltomaki P, Leach FS, Sistonen P, Pylkkanen L, Mecklin JP, Jarvinen H, Powell SM, Jen J, Hamilton SR. Clues to the pathogenesis of familial colorectal cancer. *Science* 1993; **260**: 812-816
19 **Peltomaki P**, Lothe RA, Aaltonen LA, Pylkkanen L, Nystrom-Lahti M, Seruca R, David L, Holm R, Ryberg D, Haugen A. Microsatellite instability is associated with tumors that characterize the hereditary non-polyposis colorectal carcinoma syndrome. *Cancer Res* 1993; **53**: 5853-5855
20 **Veigl ML**, Kasturi L, Olechnowicz J, Ma AH, Lutterbaugh JD, Periyasamy S, Li GM, Drummond J, Modrich PL, Sedwick WD, Markowitz SD. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc Natl Acad Sci USA* 1998; **95**: 8698-8702
21 **Kuismanen SA**, Holmberg MT, Salovaara R, de la Chapelle A, Peltomaki P. Genetic and epigenetic modification of MLH1 accounts for a major share of microsatellite-unstable colorectal cancers. *Am J Pathol* 2000; **156**: 1773-1779
22 **Holliday R**. The inheritance of epigenetic defects. *Science* 1987; **238**: 163-170
23 **Herman JG**, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003; **349**: 2042-2054
24 **Weber M**, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 2007; **39**: 457-466
25 **Linhart HG**, Lin H, Yamada Y, Moran E, Steine EJ, Gokhale S, Lo G, Cantu E, Ehrich M, He T, Meissner A, Jaenisch R. Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. *Genes Dev* 2007; **21**: 3110-3122
26 **Yoo CB**, Chuang1 JC, Byun HM, Egger G, Yang AS, Dubeau L, Long T, Laird PW, Marquez VE, Jones PA. Long-term epigenetic therapy with oral zebularine has minimal side effects and prevents intestinal tumors in mice. *Cancer Prev Res* 2008; **1**: 233-240
27 **Esteller M**. Epigenetics in cancer. *N Engl J Med* 2008; **358**: 1148-1159
28 **Mulero-Navarro S**, Esteller M. Epigenetic biomarkers for human cancer: the time is now. *Crit Rev Oncol Hematol* 2008; **68**: 1-11
29 **Ollikainen M**, Hannelius U, Lindgren CM, Abdel-Rahman WM, Kere J, Peltomaki P. Mechanisms of inactivation of MLH1 in hereditary nonpolyposis colorectal carcinoma: a novel approach. *Oncogene* 2007; **26**: 4541-4549
30 **Chen LC**, Hao CY, Chiu YS, Wong P, Melnick JS, Brotman M, Moretto J, Mendes F, Smith AP, Bennington JL, Moore D, Lee NM. Alteration of gene expression in normal-appearing colon mucosa of APC(min) mice and human cancer patients. *Cancer Res* 2004; **64**: 3694-3700
31 **Kuismanen SA**, Holmberg MT, Salovaara R, Schweizer P, Aaltonen LA, de La Chapelle A, Nystrom-Lahti M, Peltomaki P. Epigenetic phenotypes distinguish microsatellite-stable and -unstable colorectal cancers. *Proc Natl Acad Sci USA* 1999; **96**: 12661-12666
32 **Joensuu EI**, Abdel-Rahman WM, Ollikainen M, Ruosaari S, Knuutila S, Peltomaki P. Epigenetic signatures of familial cancer are characteristic of tumor type and family category. *Cancer Res* 2008; **68**: 4597-4605

33 **Toyota M**, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999; **96**: 8681-8686

34 **Ogino S**, Cantor M, Kawasaki T, Brahmandam M, Kirkner GJ, Weisenberger DJ, Campan M, Laird PW, Loda M, Fuchs CS. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut* 2006; **55**: 1000-1006

35 **Weisenberger DJ**, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006; **38**: 787-793

36 **Toyota M**, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc Natl Acad Sci USA* 2000; **97**: 710-715

37 **Teodoridis JM**, Hardie C, Brown R. CpG island methylator phenotype (CIMP) in cancer: causes and implications. *Cancer Lett* 2008; **268**: 177-186

38 **Boyle P**, Leon ME. Epidemiology of colorectal cancer. *Br Med Bull* 2002; **64**: 1-25

39 **Stewart BE**, Kleinhues P, editors. World Cancer Report. Lyon: IARC Press, 2003

40 **Willett WC**. Diet, nutrition, and avoidable cancer. *Environ Health Perspect* 1995; **103** Suppl 8: 165-170

41 **Meyerhardt JA**, Niedzwiecki D, Hollis D, Saltz LB, Hu FB, Mayer RJ, Nelson H, Whittom R, Hantel A, Thomas J, Fuchs CS. Association of dietary patterns with cancer recurrence and survival in patients with stage III colon cancer. *JAMA* 2007; **298**: 754-764

42 **Su LK**, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 1992; **256**: 668-670

43 **Pajari AM**, Oikarinen SI, Duan RD, Mutanen M. A high-beef diet alters protein kinase C isozyme expression in rat colonic mucosa. *J Nutr Biochem* 2000; **11**: 474-481

44 **Rajakangas J**, Basu S, Salminen I, Mutanen M. Adenoma growth stimulation by the trans-10, cis-12 isomer of conjugated linoleic acid (CLA) is associated with changes in mucosal NF-kappaB and cyclin D1 protein levels in the Min mouse. *J Nutr* 2003; **133**: 1943-1948

45 **Misikangas M**, Pajari AM, Paivarinta E, Mutanen M. Promotion of adenoma growth by dietary inulin is associated with increase in cyclin D1 and decrease in adhesion proteins in Min/+ mice mucosa. *J Nutr Biochem* 2005; **16**: 402-409

46 **Rajakangas J**, Pajari AM, Misikangas M, Mutanen M. Nuclear factor kappaB is downregulated and correlates with p53 in the Min mouse mucosa during an accelerated tumor growth. *Int J Cancer* 2006; **118**: 279-283

47 **Mutanen M**, Pajari AM, Oikarinen SI. Beef induces and rye bran prevents the formation of intestinal polyps in Apc(Min) mice: relation to beta-catenin and PKC isozymes. *Carcinogenesis* 2000; **21**: 1167-1173

48 **Lipkin M**, Yang K, Edelmann W, Xue L, Fan K, Risio M, Newmark H, Kucherlapati R. Preclinical mouse models for cancer chemoprevention studies. *Ann N Y Acad Sci* 1999; **889**: 14-19

49 **Yang K**, Lamprecht SA, Shinozaki H, Fan K, Yang W, Newmark HL, Kopelovich L, Edelmann W, Jin B, Gravaghi C, Augenlicht L, Kucherlapati R, Lipkin M. Dietary calcium and cholecalciferol modulate cyclin D1 expression, apoptosis, and tumorigenesis in intestine of adenomatous polyposis coli1638N/+ mice. *J Nutr* 2008; **138**: 1658-1663

50 **Choi SW**, Friso S, Dolnikowski GG, Bagley PJ, Edmondson AN, Smith DE, Mason JB. Biochemical and molecular aberrations in the rat colon due to folate depletion are age-specific. *J Nutr* 2003; **133**: 1206-1212

51 **Song J**, Medline A, Mason JB, Gallinger S, Kim YI. Effects of dietary folate on intestinal tumorigenesis in the apcMin mouse. *Cancer Res* 2000; **60**: 5434-5440

52 **Newmark HL**, Yang K, Lipkin M, Kopelovich L, Liu Y, Fan K, Shinozaki H. A Western-style diet induces benign and malignant neoplasms in the colon of normal C57Bl/6 mice. *Carcinogenesis* 2001; **22**: 1871-1875

53 **Ferguson LR**, Chavan RR, Harris PJ. Changing concepts of dietary fiber: implications for carcinogenesis. *Nutr Cancer* 2001; **39**: 155-169

54 **Jiang YH**, Lupton JR, Chang WC, Jolly CA, Aukema HM, Chapkin RS. Dietary fat and fiber differentially alter intracellular second messengers during tumor development in rat colon. *Carcinogenesis* 1996; **17**: 1227-1233

55 **Norat T**, Bingham S, Ferrari P, Slimani N, Jenab M, Mazuir M, Overvad K, Olsen A, Tjønneland A, Clavel F, Boutron-Ruault MC, Kesse E, Boeing H, Bergmann MM, Nieters A, Linseisen J, Trichopoulou A, Trichopoulos D, Tountas Y, Berrino F, Palli D, Panico S, Tumino R, Vineis P, Bueno-de-Mesquita HB, Peeters PH, Engeset D, Lund E, Skeie G, Ardanaz E, González C, Navarro C, Quirós JR, Sanchez MJ, Berglund G, Mattisson I, Hallmans G, Palmqvist R, Day NE, Khaw KT, Key TJ, San Joaquin M, Hémon B, Saracci R, Kaaks R, Riboli E. Meat, fish, and colorectal cancer risk: the European Prospective Investigation into cancer and nutrition. *J Natl Cancer Inst* 2005; **97**: 906-916

56 **Paivarinta E**, Pajari AM, Torronen R, Mutanen M. Ellagic acid and natural sources of ellagitannins as possible chemopreventive agents against intestinal tumorigenesis in the Min mouse. *Nutr Cancer* 2006; **54**: 79-83

57 **Misikangas M**, Pajari AM, Paivarinta E, Oikarinen SI, Rajakangas J, Marttinen M, Tanayama H, Torronen R, Mutanen M. Three Nordic berries inhibit intestinal tumorigenesis in multiple intestinal neoplasia/+ mice by modulating beta-catenin signaling in the tumor and transcription in the mucosa. *J Nutr* 2007; **137**: 2285-2290

58 **Mahmoud NN**, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, Newmark HL, Bertagnolli MM. Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000; **21**: 921-927

59 **Thangapazham RL**, Sharma A, Maheshwari RK. Multiple molecular targets in cancer chemoprevention by curcumin. *AAPS J* 2006; **8**: E443-E449

60 **Dashwood WM**, Carter O, Al-Fageeh M, Li Q, Dashwood RH. Lysosomal trafficking of beta-catenin induced by the tea polyphenol epigallocatechin-3-gallate. *Mutat Res* 2005; **591**: 161-172

61 **Oshima M**, Takahashi M, Oshima H, Tsutsumi M, Yazawa K, Sugimura T, Nishimura S, Wakabayashi K, Taketo MM. Effects of docosahexaenoic acid (DHA) on intestinal polyp development in Apc delta 716 knockout mice. *Carcinogenesis* 1995; **16**: 2605-2607

62 **Surh YJ**. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003; **3**: 768-780

63 **Yamada H**, Sugimura H, Tsuneyoshi T. Suppressive effect of epigallocatehin (EGCg) on DNA methylation in mice: Detection by methylation selective restriction endonuclease digestion and PCR. *J Food Agr Environ* 2005; **3**: 73-76

64 **Lee WJ**, Zhu BT. Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols. *Carcinogenesis* 2006; **27**: 269-277

65 **Fang M**, Chen D, Yang CS. Dietary polyphenols may affect DNA methylation. *J Nutr* 2007; **137**: 223S-228S

66 **Davis CD**, Uthus EO. DNA methylation, cancer susceptibility, and nutrient interactions. *Exp Biol Med* (Maywood) 2004; **229**: 988-995

67 **Kim YI**. Nutritional epigenetics: impact of folate deficiency

on DNA methylation and colon cancer susceptibility. *J Nutr* 2005; **135**: 2703-2709
68 **Wolff GL**, Kodell RL, Moore SR, Cooney CA. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. *FASEB J* 1998; **12**: 949-957
69 **Cooney CA**, Dave AA, Wolff GL. Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. *J Nutr* 2002; **132**: 2393S-2400S
70 **Fraga MF**, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M. Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci USA* 2005; **102**: 10604-10609
71 **Pietiläinen KH**, Naukkarinen J, Rissanen A, Saharinen J, Ellonen P, Keranen H, Suomalainen A, Gotz A, Suortti T, Yki-Jarvinen H, Oresic M, Kaprio J, Peltonen L. Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. *PLoS Med* 2008; **5**: e51
72 **Barker DJ**, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet* 1989; **2**: 577-580
73 **Hales CN**, Ozanne SE. The dangerous road of catch-up growth. *J Physiol* 2003; **547**: 5-10
74 **Xiao R**, Hennings LJ, Badger TM, Simmen FA. Fetal programming of colon cancer in adult rats: correlations with altered neonatal growth trajectory, circulating IGF-I and IGF binding proteins, and testosterone. *J Endocrinol* 2007; **195**: 79-87
75 **Issa JP**. Cancer prevention: epigenetics steps up to the plate. *Cancer Prev Res* 2008; **1**: 219-222

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GUIDELINES BASIC SCIENCE

Genetic mechanisms underlying the pathogenesis of tropical calcific pancreatitis

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Abstract

Chronic pancreatitis is known to be a heterogeneous disease with varied etiologies. Tropical calcific pancreatitis (TCP) is a severe form of chronic pancreatitis unique to developing countries. With growing evidence of genetic factors contributing to the pathogenesis of TCP, this review is aimed at compiling the available information in this field. We also propose a two hit model to explain the sequence of events in the pathogenesis of TCP.

Key words: Chronic pancreatitis; Tropical calcific pancreatitis; Fibrocalculous pancreatic diabetes; Complex disease; Candidate gene analysis

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INTRODUCTION

Pancreatitis is a heterogeneous disease with varied etiologies, defined as an inflammatory disease of the pancreas leading to morphologic changes that typically cause pain and/or loss of function. Chronic pancreatitis (CP), [Online Mendelian inheritance in man (OMIM) 167800], is a continuing inflammatory disease which eventually leads to morphologic changes characterized by irreversible destruction and fibrosis of the exocrine parenchyma, leading to exocrine pancreatic insufficiency and progressive endocrine failure leading to diabetes. Histologic changes from the normal pancreatic architecture include irregular fibrosis, acinar cell loss, islet cell loss and inflammatory cell infiltrates, and distorted and blocked ducts[1]. Thus expert "state-of-the-science" reviewers conceded that "chronic pancreatitis remains an enigmatic process of uncertain pathogenesis, unpredictable clinical course, and unclear treatment"[2]. In most developed countries, alcohol causes about 60%-70% of the cases of chronic pancreatitis in male patients, and unknown causes are responsible for 25% of cases, termed as idiopathic chronic pancreatitis (ICP). Tropical calcific pancreatitis (TCP, OMIM 608189) is a juvenile form of chronic calcific non alcoholic pancreatitis, seen almost exclusively in developing countries of the tropical world[3]. In the most simple of terms, tropical calcific pancreatitis has been described as a disease with "pain in childhood, diabetes in puberty and death at the prime of life"[4].

TCP patients in former years were mostly children, adolescents, or sometimes young adults, who had common characteristics of malnutrition, deficiency signs, a cyanotic hue of enlarged lips, bilaterally enlarged parotid glands, a pot belly, and sometimes pedal edema. However, the clinical features and presentation of tropical pancreatitis has changed over the past 50 years with an older age of onset; severe malnutrition being uncommon with many patients being of ideal body weight which is attributed to improved nutritional status[5-8].

The cardinal manifestations of TCP are recurrent abdominal pain in childhood, followed by onset of diabetes mellitus a few years later. Prevalence of pancreatic calculi in TCP is nearly 90%, which is much higher than in alcoholic pancreatitis (30%)[9]. Pancreatic calculi varying in size and shape are demonstrable

throughout the markedly dilated main duct forming a ductogram and in some cases even in the dilated ductules mimicking a pancreatogram[9,10]. Early reports on TCP identified patients only in the late stages of the disease when extreme emaciation and other obvious clinical signs of protein malnutrition, such as bilateral parotid gland enlargement as well as skin and hair changes of kwashiorkor, dominated the clinical picture[11]. A recent population based study in southern India has shown the prevalence of TCP to be 0.02% in the general population[12]. Histopathological changes include dilation of the main pancreatic duct, intralobular fibrosis in early and interacinar fibrosis in later stages[13]. Unlike other forms of CP, the diabetes secondary to TCP has been given the unique name of 'fibrocalculous pancreatic diabetes' (FCPD).

ETIOPATHOGENESIS OF TCP

Etiopathogenic mechanisms of TCP are still unclear. Based on the observation that TCP almost exclusively affects the poor population of developing nations, malnutrition was strongly suspected to be a major etiologic factor. The role of under-nutrition in the etiology of TCP has been extensively reviewed[14-17]. However, recent observations suggest that malnutrition could be the effect rather than the cause of the disease. The geographical distribution of TCP coincides with the areas of consumption of cassava (Tapioca, *Manihot esculenta*), which is the staple diet of poor people in Kerala, a state in India. Cyanogen toxicity in the presence of malnutrition and antioxidant deficiency has been proposed as an ideal setting for free radical injury[18]. However, TCP is prevalent in many parts of India and Africa where cassava is not consumed and is not seen in West African populations consuming a high cassava diet[19]. A study on rats fed with a cassava diet for one year did not produce either pancreatitis or diabetes[20]. Thus it is unlikely that cassava ingestion can explain the majority of cases of TCP seen world wide and the current opinion is that cyanogen toxicity is not relevant in its etiopathogenesis. The contribution of dietary factors like proteins, and carbohydrates is not clear. The micronutrient deficiency-induced free radical hypothesis[21,22] remains to be proven and certainly merits further studies.

GENETICS OF TCP

It had been hypothesized about a century ago that the first important step in the development of pancreatitis is the inappropriate activation of trypsinogen in the pancreas[23,24]. Three different trypsinogens; cationic, anionic and meso, representing 23.1%, 16% and 0.5% of total pancreatic secretory proteins respectively, have been described in human pancreatic juice[25]. Normally, after trypsinogen is secreted into the duodenum it becomes active due to the action of an intestinal endopeptidase called enterokinase at the Lys15-Ile16 peptide bond, releasing the N-terminal octapeptide called trypsinogen activation peptide (TAP). It is thought that generally about 5% of trypsinogens get activated within the normal pancreas, but the pancreas has several safety mechanisms to cope with the premature activation of these enzymes, which would otherwise lead to indiscriminate proteolysis (autodigestion)[26].

Trypsin is known to lose its activity spontaneously by autolysis at the initial hydrolytic point of trypsin at Arg122-Lys123, which renders it more susceptible to further degradation[27]. A~6 kDa protein termed pancreatic secretory trypsin inhibitor (PSTI) or serine protease inhibitor Kazal type I (*SPINK1*, OMIM 167790) is present in the secretory granules of acinar cells which binds to the active site of trypsin in a 1:1 ratio and inhibits tryptic activities. Other safety mechanisms are the presence of trypsin inhibitors in plasma including α1-antitrypsin and β2-microglobulin, which inhibit the trypsin that leaks into the interstitial space around the pancreas[26]. It has been hypothesized that the primary mechanism to prevent trypsin injury inside the acinar cell is to maintain calcium at low levels[28]. Trypsinogen activation and trypsin survival are known to be regulated by calcium. Once trypsinogen is secreted into the duct, the calcium-dependent mechanisms utilized by the acinar cell for protection from trypsin become irrelevant because the calcium levels in the duct are quite high. Instead, the duct is protected through maintenance of an alkaline pH and by rapid flushing of the zymogens and prematurely activated enzymes out of the pancreas and into the duodenum[29]. Thus, trypsinogen plays a key role in the initiation of pancreatitis by evading the protective mechanisms leading to autodigestion of pancreas.

A high-density map of the human genome based on polymorphic simple tandem repeat (STR) markers and familial linkage analysis on several affected and unaffected individuals in several generations made it possible to identify an hereditary pancreatitis (HP) gene locus on chromosome 7q35[30,31]. Subsequently a mutation (365G>A) leading to arginine to histidine substitution at 122 position (R122H) in cationic trypsinogen gene [protease, serine, 1 (trypsin 1)(*PRSS1*), OMIM 276000], was found to be associated with hereditary pancreatitis[32]. Subsequent studies reported other *PRSS1* alterations including A16V, N29T, R116C, and R122C, as well as several others, in families with suspected hereditary pancreatitis or in patients without a family history (www.uni-leipzig.de/pancreasmutation)[33]. The current model of *PRSS1* mutations suggests that the identified mutations cause enhanced auto-activation of trypsinogen to trypsin or prevent prematurely activated trypsin from being inactivated by autolysis.

Familial aggregation is seen in about 8% of TCP patients. In some families, there has been evidence of vertical transmission of TCP from patients to offspring, while in others horizontal distribution of the disease among siblings was reported[34]. Familial aggregations suggest a genetic etiology for TCP. However, on screening known susceptibility factor, *PRSS1*, reported to be associated with HP and CP in Western populations, no association with TCP was found[35-37]. Instead, the

inhibitor of trypsinogen called *SPINK1* has been reported to be strongly associated with TCP[38,39]. An A>G transition at 101 nucleotide position in the *SPINK1* gene leading to substitution of asparagine by serine at codon 34 (N34S) has been reported with its highest frequency (approximate 46%) found so far in the Indian population[37]. Similar associations with varying strength have been reported by several studies, establishing *SPINK1* as a strong candidate for contributing to the pathogenesis of TCP[40,41]. Loss of function mutations in protease inhibitor *SPINK1* is thought to result in sustained "super-trypsin" activity. However, no genotype-phenotype correlation was found in patients carrying the N34S *SPINK1* mutation in homozygous or heterozygous states[42] and a wide variability has been reported in the pattern of inheritance[40]. Functional studies with human recombinant N34S *SPINK1* did not show altered trypsin inhibitor capacity or secretion[43-45]. An animal model deficient of Spink3, the murine orthologue of human *SPINK1*, showed progressive disappearance of acinar cells due to autophagic cell death and impaired regeneration. Thus, it might be surmised that *SPINK1* plays an essential role in the maintenance of integrity and regeneration of acinar cells[46]. Nevertheless, pathogenic mechanisms of N34S remain obscure. However, N34S has been observed to be in complete linkage disequilibrium with four intronic variants, 56-37T>C, 87+268A>g, 195-604G>A, 195-66_-65insTTTT[47], one of which may be pathogenic. Thus, in spite of being the strongest predictor and an important risk factor in the pathogenesis of TCP, the mechanism of N34S *SPINK1* still remains elusive.

Mutations in anionic trypsinogen [protease, serine, 2 (trypsin 2) (*PRSS2*), OMIM 601564] have been hypothesized to cause the disease by a mechanism similar to that of *PRSS1*. Earlier studies by various groups in ICP and TCP patients did not find associated polymorphisms in *PRSS2*[48,49]. However, a glycine to arginine change at codon 191 in *PRSS2* screened in a European population has been demonstrated to play a protective role against chronic pancreatitis[50]. Functional studies on purified recombinant G191R protein revealed that generation of a novel tryptic cleavage site within the mutated gene product makes the enzyme hypersensitive to autocatalytic proteolysis, thus playing a protective role in chronic pancreatitis. However, data from a study by Chandak's group (manuscript under review) suggests that this variant may not have a significant role to play in the Indian population. A very low allele frequency in the control populations and a comparable frequency in TCP patients are suggestive of the variant allele being neutral to natural selection. This could possibly be due to the dietary patterns marked by low protein consumption.

An association of Cystic fibrosis transmembrane regulator (*CFTR*, OMIM 602421) gene with alcoholic pancreatitis and ICP has been reported, where about 13.4%[51] and 25.9%[52] of patients in two studies were shown to carry at least one mutation in the gene. A study by Noone *et al*[53] revealed association of *CFTR* mutations with ICP and a possibility of its interaction with *PRSS1* and *SPINK1* mutations in western populations. However, the frequency of *CFTR* mutations was found to be lower in TCP patients[54], and needs to be studied in a larger group of patients.

Previous studies with synthetic substrates demonstrated a similarity between cathepsin B (*CTSB*, OMIM 116810) and trypsin in their specificity towards synthetic substrates. This made it of interest to observe if cathepsin B might activate trypsinogen. There is evidence to suggest that partially purified beef spleen cathepsin B activates trypsinogen to a trypsin-like product. Studies on native and recombinant cationic trypsinogen assigned a central role of cathepsin B in the development of different forms of pancreatitis[55]. It was recently shown that polymorphisms in *CTSB* are associated with TCP[56]. Mutations in the propeptide region of the *CTSB* gene like L26V and S53G have been found to be associated with TCP and it has been hypothesized that inappropriate localization of cathepsin B protein in zymogen granules due to these mutations could lead to premature activation of trypsinogen. This not only suggests an important role for *CTSB* polymorphisms in TCP, but also advocates emphasis on factors likely to change the pH or alter the intracellular calcium levels.

An important feature of TCP is the high incidence of pancreatic calcification and stone formation. It has been suggested that lithostathine C [coded by regenerating islet-derived protein (*Reg*) genes], a major proteic component of pancreatic stone in patients with alcoholic calcifying chronic pancreatitis, could promote the nucleation of calcite crystals or may prevent pancreatic lithiasis by inhibiting calcite crystal nucleation and growth in the pancreatic juice. With suggestions that it might help in preventing the harmful activation of protease precursors in the pancreatic juice, it was thought to be a logical assumption that mutations in this gene could lead to pancreatitis and calcification[57]. Exons of *Reg1*α gene (OMIM 167770) were screened for associated polymorphisms, but no association has been found so far[58,59]. As the protein is known to be down-regulated in TCP patients, a recent study screened the gene including the putative promoter and intronic regions, but did not find a significant association with TCP[60]. *Reg1*α is highly represented in human pancreatic secretions unlike *Reg1*β (OMIM 167771), which is 87% homologous to *Reg1*α and is not extensively studied and remains to be characterized[61].

Progression to diabetes, called fibro-calculous pancreatic diabetes (FCPD), which takes place in a majority of TCP patients, is another important feature of TCP, but the nature of the diabetes is controversial. A recent study, hypothesized that investigating a known susceptibility factor for T1D or T2D can help in understanding the type and mechanism of diabetes in FCPD patients. In this study type 2 diabetes (T2D) associated polymorphisms in transcription factor 7 like protein 2 (*TCF7L2,* OMIM 602228) were screened in TCP and FCPD patients. Although no association was found with FCPD independently, data suggested that the

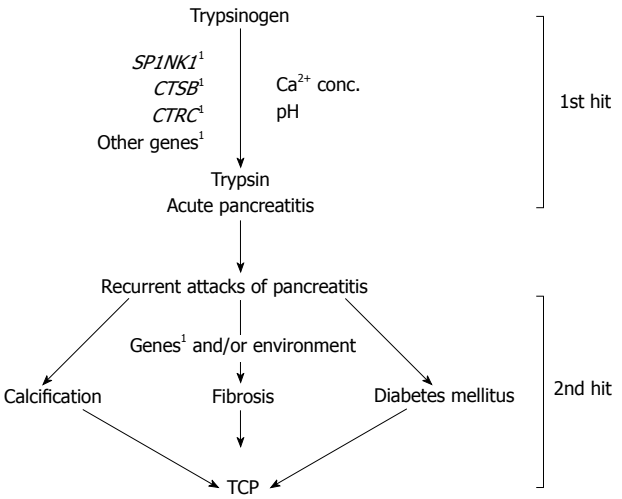

Figure 1 Two hit model for the pathogenesis of TCP. First hit contributing to the pathogenesis of TCP is likely to be loss of balance between activation events and degradation of active trypsin leading to presence of persistent "super-trypsin" within the acinar cell, which could occur due to mutations in one or more genes like *SPINK1, CTSB, CTRC*, other yet unidentified genes, resulting in inflammation. Presence of additional genetic and/or environmental factors, which constitute the second hit, may lead to one or more phenotypes such as stone formation, fibrosis, and/or diabetes mellitus. [1]Mutation in genes.

polymorphisms in *TCF7L2* may interact with *SPINK1* and *CTSB* mutations and cause FCPD[62].

Increased accumulation of extracellular matrix is a histological characteristic of chronic pancreatitis that results in pancreatic fibrosis (Haber *et al*, 1999). Angiotensin converting enzyme (ACE, OMIM 106180), a zinc metallopeptidase which is a key enzyme of the renin-angiotensin system (RAS) and is known to proliferate hepatic stellate cells, has been hypothesized to play a role in pancreatic fibrosis in TCP patients. A polymorphism within intron 16 (g.11417_11704del287) of the ACE gene is strongly related to the circulating enzyme levels in a dose dependent manner. However, no association of this polymorphism has been found with TCP[63].

Genetic and functional data from a recent study by Rosendahl *et al*[64] identified chymotrypsin C (*CTRC*, OMIM 601405) as a new pancreatitis-associated gene and discovered that loss-of-function alterations in the gene predispose to pancreatitis by diminishing its protective trypsin-degrading activity. The same was shown to be true with TCP patients. Their observations provided support for the trypsin-dependent pathogenic model of chronic pancreatitis in humans by demonstrating that trypsin-trypsinogen degradation by *CTRC* is an important mechanism for maintaining the physiological protease-antiprotease balance in the pancreas. Copy number variations, i.e. triplication of a 605 kilobase segment containing the *PRSS1* and *PRSS2* genes have been reported in hereditary pancreatitis patients[65]. A study by Masson *et al*[66] revealed the molecular basis of 6% of young ICP patients demonstrating chronic pancreatitis to be a genomic disorder. However, no copy number variations were found in TCP patients to provide evidence, showing that trypsinogen gene mutations do not play an important role in the pathogenesis of TCP in the Indian population.

Mutations involving the calcium sensing receptor (*CASR*, OMIM 601199) have been suggested to increase the risk of chronic pancreatitis (CP), since high intracellular levels of calcium activate trypsinogen within the acinar cells. A combination of *CASR* and *SPINK1* gene mutations has been proposed to predispose to idiopathic CP[67]. A study by Murugaian *et al*[68] identified 4 novel *CASR* mutations in TCP patients and concluded that the risk of disease may be further increased if there is an associated *SPINK1* mutation.

CONCLUSION

In conclusion, all the established mutations in the cationic trypsinogen gene, including the copy number polymorphism, are not a common cause of tropical calcific pancreatitis in the Indian population[37,66]. The model for etiopathogenesis of TCP emerging from the available information is presented in Figure 1. Many aspects of TCP remain unclear. What triggers intra-pancreatic trypsin activation, and in the presence of an intact autolysis site how is it maintained in an active state? Are the various manifestations of TCP, such as calcification, ketosis resistant diabetes mellitus, pancreatic cancer and fibrosis, consequences of a proteolytic cascade of prematurely activated trypsin? Since TCP is a complex disease, in addition to candidate gene analysis which has undoubtedly been influential, there is a necessity for a more comprehensive and holistic approach to understand its etiopathogenesis, to help early detection and discover possible treatment. The role of environmental factors as disease modifiers cannot be undermined. An in-depth study of the contribution of dietary- and lifestyle-related factors, and their association with genetic variants would yield interesting leads.

REFERENCES

1 **Klöppel G**, Maillet B. Chronic pancreatitis: evolution of the disease. *Hepatogastroenterology* 1991; **38**: 408-412
2 **Steer ML**, Waxman I, Freedman S. Chronic pancreatitis. *N Engl J Med* 1995; **332**: 1482-1490
3 **Barman KK**, Premalatha G, Mohan V. Tropical chronic pancreatitis. *Postgrad Med J* 2003; **79**: 606-615
4 **Geevarghese PJ**. Calcific Pancreatitis. Bombay: Vargheese Publishing House, 1985
5 **Viswanathan M**. Pancreatic Diabetes in India: an overview. In: Podolsky S, Viswanathan M, eds. Secondary Diabetes: the spectrum of the diabetic syndromes. New York: Raven Press, 1980: 105-116
6 **Zuidema PJ**. Calcification and cirrhosis of the pancreas in patients with deficient nutrition. *Doc Med Geogr Trop* 1955; **7**: 229-251
7 **Pitchumoni CS**, Mohan V. Pancreatitis: Juvenile Tropical Pancreatitis. In: Walker WA, Goulet O, Kleinman RE, Sherman PM, Shneider BL, Sanderson IR, editors. Pediatric Gastrointestinal Disease: Pathophysiology, Diagnosis, Management. Hamilton: BC Decker, 2004; 1598-1605
8 **Mohan V**, Premalatha G, Pitchumoni CS. Tropical chronic pancreatitis: an update. *J Clin Gastroenterol* 2003; **36**: 337-346
9 **Balakrishnan V**. Chronic calcific pancreatitis in the tropics.

Indian J Gastroenterol 1984; **3**: 65-67
10 **Pitchumoni CS**, Viswanathan KV, Gee Varghese PJ, Banks PA. Ultrastructure and elemental composition of human pancreatic calculi. *Pancreas* 1987; **2**: 152-158
11 **Zuidema PJ**. Cirrhosis and disseminated calcification of the pancreas in patients with malnutrition. *Trop Geogr Med* 1959; **11**: 70-74
12 **Mohan V**, Farooq S, Deepa M. Prevalence of fibrocalculous pancreatic diabetes in Chennai in South India. *JOP* 2008; **9**: 489-492
13 **Reddy DN**. Tropical pancreatitis - The Indian experience. 8th World Congress of the International Gastro-Surgical Club; 1998 Apr 15-18; Strasbourg, France. Bologna: Litosei-Rastignano, 1998: 249-253
14 **Rao RH**. The role of undernutrition in the pathogenesis of diabetes mellitus. *Diabetes Care* 1984; **7**: 595-601
15 **Pitchumoni CS**. Special problems of tropical pancreatitis. *Clin Gastroenterol* 1984; **13**: 941-959
16 **Rao RH**, Yajnik CS. Commentary: time to rethink malnutrition and diabetes in the tropics. *Diabetes Care* 1996; **19**: 1014-1017
17 **Mohan V**, Pitchumoni CS. Tropical chronic pancreatitis. In: Berger HG, Warshaw AL, Buchler MW, eds. The Pancreas, 1st ed. London: Blackwell Science, 1998: 688-697
18 **McMillan DE**, Geevarghese PJ. Dietary cyanide and tropical malnutrition diabetes. *Diabetes Care* 1979; **2**: 202-208
19 **Teuscher T**, Baillod P, Rosman JB, Teuscher A. Absence of diabetes in a rural West African population with a high carbohydrate/cassava diet. *Lancet* 1987; **1**: 765-768
20 **Mathangi DC**, Deepa R, Mohan V, Govindarajan M, Namasivayam A. Long-term ingestion of cassava (tapioca) does not produce diabetes or pancreatitis in the rat model. *Int J Pancreatol* 2000; **27**: 203-208
21 **Braganza JM**. The pancreas. In: Pounder RG, ed. Recent advances in Gastroenterology. London: Churchill Livingstone, 1988: 251–280
22 **Braganza JM**. Free radicals and pancreatitis. In: Rice-Evans C, Dormandy T, eds. Free radicals: chemistry, pathology and medicine. London: Richelieu Press, 1988: 357–381
23 **Chiari H**. Über Selbstverdauung des menschlichen Pankreas. *Zeitschrift für Heilkunde* 1896; **17**: 69-96
24 **Steer ML**, Meldolesi J. The cell biology of experimental pancreatitis. *N Engl J Med* 1987; **316**: 144-150
25 **Scheele GA**. Two-dimensional gel analysis of soluble proteins. Charaterization of guinea pig exocrine pancreatic proteins. *J Biol Chem* 1975; **250**: 5375-5385
26 **Gaboriaud C**, Serre L, Guy-Crotte O, Forest E, Fontecilla-Camps JC. Crystal structure of human trypsin 1: unexpected phosphorylation of Tyr151. *J Mol Biol* 1996; **259**: 995-1010
27 **Naruse S**, Kitagawa M, Ishiguro H. Molecular understanding of chronic pancreatitis: a perspective on the future. *Mol Med Today* 1999; **5**: 493-499
28 **Whitcomb DC**. Mechanisms of disease: Advances in understanding the mechanisms leading to chronic pancreatitis. *Nat Clin Pract Gastroenterol Hepatol* 2004; **1**: 46-52
29 **Sutton R**, Criddle D, Raraty MG, Tepikin A, Neoptolemos JP, Petersen OH. Signal transduction, calcium and acute pancreatitis. *Pancreatology* 2003; **3**: 497-505
30 **Le Bodic L**, Bignon JD, Raguénès O, Mercier B, Georgelin T, Schnee M, Soulard F, Gagne K, Bonneville F, Muller JY, Bachner L, Férec C. The hereditary pancreatitis gene maps to long arm of chromosome 7. *Hum Mol Genet* 1996; **5**: 549-554
31 **Whitcomb DC**, Preston RA, Aston CE, Sossenheimer MJ, Barua PS, Zhang Y, Wong-Chong A, White GJ, Wood PG, Gates LK Jr, Ulrich C, Martin SP, Post JC, Ehrlich GD. A gene for hereditary pancreatitis maps to chromosome 7q35. *Gastroenterology* 1996; **110**: 1975-1980
32 **Whitcomb DC**, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gates LK Jr, Amann ST, Toskes PP, Liddle R, McGrath K, Uomo G, Post JC, Ehrlich GD. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet* 1996; **14**: 141-145
33 **Teich N**, Rosendahl J, Tóth M, Mössner J, Sahin-Tóth M. Mutations of human cationic trypsinogen (PRSS1) and chronic pancreatitis. *Hum Mutat* 2006; **27**: 721-730
34 **Mohan V**, Chari ST, Hitman GA, Suresh S, Madanagopalan N, Ramachandran A, Viswanathan M. Familial aggregation in tropical fibrocalculous pancreatic diabetes. *Pancreas* 1989; **4**: 690-693
35 **Rossi L**, Whitcomb DC, Ehrlich GD, Gorry MC, Parvin S, Sattar S, Ali L, Azad Khan AK, Gyr N. Lack of R117H mutation in the cationic trypsinogen gene in patients with tropical pancreatitis from Bangladesh. *Pancreas* 1998; **17**: 278-280
36 **Hassan Z**, Mohan V, McDermott MF, Ali L, Ogunkolade WB, Aganna E, Cassell PG, Deepa R, Khan AK, Hitman GA. Pancreatitis in fibrocalculous pancreatic diabetes mellitus is not associated with common mutations in the trypsinogen gene. *Diabetes Metab Res Rev* 2000; **16**: 454-457
37 **Chandak GR**, Idris MM, Reddy DN, Bhaskar S, Sriram PV, Singh L. Mutations in the pancreatic secretory trypsin inhibitor gene (PSTI/SPINK1) rather than the cationic trypsinogen gene (PRSS1) are significantly associated with tropical calcific pancreatitis. *J Med Genet* 2002; **39**: 347-351
38 **Rossi L**, Pfützer RH, Parvin S, Ali L, Sattar S, Kahn AK, Gyr N, Whitcomb DC. SPINK1/PSTI mutations are associated with tropical pancreatitis in Bangladesh. A preliminary report. *Pancreatology* 2001; **1**: 242-245
39 **Schneider A**, Suman A, Rossi L, Barmada MM, Beglinger C, Parvin S, Sattar S, Ali L, Khan AK, Gyr N, Whitcomb DC. SPINK1/PSTI mutations are associated with tropical pancreatitis and type II diabetes mellitus in Bangladesh. *Gastroenterology* 2002; **123**: 1026-1030
40 **Bhatia E**, Choudhuri G, Sikora SS, Landt O, Kage A, Becker M, Witt H. Tropical calcific pancreatitis: strong association with SPINK1 trypsin inhibitor mutations. *Gastroenterology* 2002; **123**: 1020-1025
41 **Hassan Z**, Mohan V, Ali L, Allotey R, Barakat K, Faruque MO, Deepa R, McDermott MF, Jackson AE, Cassell P, Curtis D, Gelding SV, Vijayaravaghan S, Gyr N, Whitcomb DC, Khan AK, Hitman GA. SPINK1 is a susceptibility gene for fibrocalculous pancreatic diabetes in subjects from the Indian subcontinent. *Am J Hum Genet* 2002; **71**: 964-968
42 **Witt H**, Luck W, Hennies HC, Classen M, Kage A, Lass U, Landt O, Becker M. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. *Nat Genet* 2000; **25**: 213-216
43 **Kuwata K**, Hirota M, Shimizu H, Nakae M, Nishihara S, Takimoto A, Mitsushima K, Kikuchi N, Endo K, Inoue M, Ogawa M. Functional analysis of recombinant pancreatic secretory trypsin inhibitor protein with amino-acid substitution. *J Gastroenterol* 2002; **37**: 928-934
44 **Király O**, Boulling A, Witt H, Le Maréchal C, Chen JM, Rosendahl J, Battaggia C, Wartmann T, Sahin-Tóth M, Férec C. Signal peptide variants that impair secretion of pancreatic secretory trypsin inhibitor (SPINK1) cause autosomal dominant hereditary pancreatitis. *Hum Mutat* 2007; **28**: 469-476
45 **Boulling A**, Le Maréchal C, Trouvé P, Raguénès O, Chen JM, Férec C. Functional analysis of pancreatitis-associated missense mutations in the pancreatic secretory trypsin inhibitor (SPINK1) gene. *Eur J Hum Genet* 2007; **15**: 936-942
46 **Ohmuraya M**, Hirota M, Araki M, Mizushima N, Matsui M, Mizumoto T, Haruna K, Kume S, Takeya M, Ogawa M, Araki K, Yamamura K. Autophagic cell death of pancreatic acinar cells in serine protease inhibitor Kazal type 3-deficient mice. *Gastroenterology* 2005; **129**: 696-705
47 **Witt H**, Luck W, Hennies HC, Classen M, Kage A, Lass U, Landt O, Becker M. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. *Nat Genet* 2000; **25**: 213-216
48 **Chen JM**, Audrezet MP, Mercier B, Quere I, Ferec C. Exclusion of anionic trypsinogen and mesotrypsinogen involvement in hereditary pancreatitis without cationic

trypsinogen gene mutations. *Scand J Gastroenterol* 1999; **34**: 831-832

49 **Idris MM**, Bhaskar S, Reddy DN, Mani KR, Rao GV, Singh L, Chandak GR. Mutations in anionic trypsinogen gene are not associated with tropical calcific pancreatitis. *Gut* 2005; **54**: 728-729

50 **Witt H**, Sahin-Tóth M, Landt O, Chen JM, Kähne T, Drenth JP, Kukor Z, Szepessy E, Halangk W, Dahm S, Rohde K, Schulz HU, Le Maréchal C, Akar N, Ammann RW, Truninger K, Bargetzi M, Bhatia E, Castellani C, Cavestro GM, Cerny M, Destro-Bisol G, Spedini G, Eiberg H, Jansen JB, Koudova M, Rausova E, Macek M Jr, Malats N, Real FX, Menzel HJ, Moral P, Galavotti R, Pignatti PF, Rickards O, Spicak J, Zarnescu NO, Böck W, Gress TM, Friess H, Ockenga J, Schmidt H, Pfützer R, Löhr M, Simon P, Weiss FU, Lerch MM, Teich N, Keim V, Berg T, Wiedenmann B, Luck W, Groneberg DA, Becker M, Keil T, Kage A, Bernardova J, Braun M, Güldner C, Halangk J, Rosendahl J, Witt U, Treiber M, Nickel R, Férec C. A degradation-sensitive anionic trypsinogen (PRSS2) variant protects against chronic pancreatitis. *Nat Genet* 2006; **38**: 668-673

51 **Sharer N**, Schwarz M, Malone G, Howarth A, Painter J, Super M, Braganza J. Mutations of the cystic fibrosis gene in patients with chronic pancreatitis. *N Engl J Med* 1998; **339**: 645-652

52 **Cohn JA**, Friedman KJ, Noone PG, Knowles MR, Silverman LM, Jowell PS. Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. *N Engl J Med* 1998; **339**: 653-658

53 **Noone PG**, Zhou Z, Silverman LM, Jowell PS, Knowles MR, Cohn JA. Cystic fibrosis gene mutations and pancreatitis risk: relation to epithelial ion transport and trypsin inhibitor gene mutations. *Gastroenterology* 2001; **121**: 1310-1319

54 **Bhatia E**, Durie P, Zielenski J, Lam D, Sikora SS, Choudhuri G, Tsui LC. Mutations in the cystic fibrosis transmembrane regulator gene in patients with tropical calcific pancreatitis. *Am J Gastroenterol* 2000; **95**: 3658-3659

55 **Szilágyi L**, Kénesi E, Katona G, Kaslik G, Juhász G, Gráf L. Comparative in vitro studies on native and recombinant human cationic trypsins. Cathepsin B is a possible pathological activator of trypsinogen in pancreatitis. *J Biol Chem* 2001; **276**: 24574-24580

56 **Mahurkar S**, Idris MM, Reddy DN, Bhaskar S, Rao GV, Thomas V, Singh L, Chandak GR. Association of cathepsin B gene polymorphisms with tropical calcific pancreatitis. *Gut* 2006; **55**: 1270-1275

57 **De Reggi M**, Gharib B. Protein-X, Pancreatic Stone-, Pancreatic thread-, reg-protein, P19, lithostathine, and now what? Characterization, structural analysis and putative function(s) of the major non-enzymatic protein of pancreatic secretions. *Curr Protein Pept Sci* 2001; **2**: 19-42

58 **Hawrami K**, Mohan V, Bone A, Hitman GA. Analysis of islet regenerating (reg) gene polymorphisms in fibrocalculous pancreatic diabetes. *Pancreas* 1997; **14**: 122-125

59 **Boonyasrisawat W**, Pulsawat P, Yenchitsomanus PT, Vannasaeng S, Pramukkul P, Deerochanawong C, Sriussadaporn S, Ploybutr S, Pasurakul T, Banchuin N. Analysis of the reg1alpha and reg1beta gene transcripts in patients with fibrocalculous pancreatopathy. *Southeast Asian J Trop Med Public Health* 2002; **33**: 365-372

60 **Mahurkar S**, Bhaskar S, Reddy DN, Rao GV, Chandak GR. Comprehensive screening for reg1alpha gene rules out association with tropical calcific pancreatitis. *World J Gastroenterol* 2007; **13**: 5938-5943

61 **Sanchez D**, Figarella C, Marchand-Pinatel S, Bruneau N, Guy-Crotte O. Preferential expression of reg I beta gene in human adult pancreas. *Biochem Biophys Res Commun* 2001; **284**: 729-737

62 **Mahurkar S**, Bhaskar S, Reddy DN, Prakash S, Rao GV, Singh SP, Thomas V, Chandak GR. TCF7L2 gene polymorphisms do not predict susceptibility to diabetes in tropical calcific pancreatitis but may interact with SPINK1 and CTSB mutations in predicting diabetes. *BMC Med Genet* 2008; **9**: 80

63 **Bhaskar S**, Reddy DN, Mahurkar S, Rao GV, Singh L, Chandak GR. Lack of significant association of an insertion/deletion polymorphism in the angiotensin converting enzyme (ACE) gene with tropical calcific pancreatitis. *BMC Gastroenterol* 2006; **6**: 42

64 **Rosendahl J**, Witt H, Szmola R, Bhatia E, Ozsvári B, Landt O, Schulz HU, Gress TM, Pfützer R, Löhr M, Kovacs P, Blüher M, Stumvoll M, Choudhuri G, Hegyi P, te Morsche RH, Drenth JP, Truninger K, Macek M Jr, Puhl G, Witt U, Schmidt H, Büning C, Ockenga J, Kage A, Groneberg DA, Nickel R, Berg T, Wiedenmann B, Bödeker H, Keim V, Mössner J, Teich N, Sahin-Tóth M. Chymotrypsin C (CTRC) variants that diminish activity or secretion are associated with chronic pancreatitis. *Nat Genet* 2008; **40**: 78-82

65 **Le Maréchal C**, Masson E, Chen JM, Morel F, Ruszniewski P, Levy P, Férec C. Hereditary pancreatitis caused by triplication of the trypsinogen locus. *Nat Genet* 2006; **38**: 1372-1374

66 **Masson E**, Le Maréchal C, Chandak GR, Lamoril J, Bezieau S, Mahurkar S, Bhaskar S, Reddy DN, Chen JM, Férec C. Trypsinogen copy number mutations in patients with idiopathic chronic pancreatitis. *Clin Gastroenterol Hepatol* 2008; **6**: 82-88

67 **Felderbauer P**, Klein W, Bulut K, Ansorge N, Dekomien G, Werner I, Epplen JT, Schmitz F, Schmidt WE. Mutations in the calcium-sensing receptor: a new genetic risk factor for chronic pancreatitis? *Scand J Gastroenterol* 2006; **41**: 343-348

68 **Murugaian EE**, Premkumar RM, Radhakrishnan L, Vallath B. Novel mutations in the calcium sensing receptor gene in tropical chronic pancreatitis in India. *Scand J Gastroenterol* 2008; **43**: 117-121

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REVIEW

Breastfeeding and genetic factors in the etiology of inflammatory bowel disease in children

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Abstract

Inflammatory bowel disease is a chronic, debilitating disorder of the gastrointestinal tract. The etiology of inflammatory bowel disease has not been elucidated, but is thought to be multifactorial with both environmental and genetic influences. A large body of research has been conducted to elucidate the etiology of inflammatory bowel disease. This article reviews this literature, emphasizing the studies of breastfeeding and the studies of genetic factors, particularly NOD2 polymorphisms.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Etiology; Risk factors; Protective factors; NOD2/CARD15; Single nucleotide polymorphisms

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INTRODUCTION

Inflammatory bowel disease (IBD) is an idiopathic condition characterized by chronic destructive inflammation of the gastrointestinal tract. The morbidity of IBD, particularly in younger patients, can be considerable and may include effects on growth and development, reproductive health, education, employment, and psychological health. The pathogenesis of IBD is thought to be a complex interaction between genetic predisposition and inappropriate activation of the mucosal immune system driven by the presence of enteric flora and resulting in tissue injury[1-3]. Genetic factors have been the subject of intense investigation and, at least in some cases, may be involved in inappropriate activation of the mucosal immune system. Discerning other factors that influence the activation of the mucosal immune system or the distribution of enteric flora present in those at risk for IBD is paramount in lessening the impact of IBD, a debilitating condition that affects children and adults throughout the world. One factor that may be important in the pathogenesis of IBD is breastfeeding. Breastfeeding is a protective factor for the development of several chronic disorders[4]. The intent of this article is to review factors involved in the development of IBD in children, with particular emphasis on genetic factors and breastfeeding.

BACKGROUND

IBD is generally considered to include two major disorders, Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are similar conditions, but most experts consider them separate diseases[2,3]. This distinction might have important therapeutic implications. In the individual patient, CD and UC can usually be distinguished on the basis of clinical features (Table 1) and laboratory manifestations, as well as radiographic, endoscopic, and histological features.

Table 1 Clinical and epidemiological features of Crohn's disease and ulcerative colitis

	CD	UC
Region of involvement	Any portion of gastrointestinal tract	Rectum and colon
Typical region of involvement	Ileum and colon	Rectum and extending proximally
Nature of inflammatory process	Segmental, transmural	Continuous, limited to mucosa
Extraintestinal manifestations	Oral aphthous ulcers, peripheral arthritis, erythema nodosum, digital clubbing, episcleritis, renal stones, and gallstones	Pyoderma gangrenosum, sclerosing cholangitis, chronic active hepatitis, and ankylosing spondylitis
Age at presentation	Bimodal; 1st peak in late teens; 2nd peak in late adulthood	Bimodal; 1st peak in late teens; 2nd peak in late adulthood
Gender difference	Women are slightly more likely than men to develop CD	Men are slightly more likely than women to develop UC

In some cases, a definitive diagnosis cannot initially be made. These patients are diagnosed with indeterminate colitis until a definitive diagnosis can be determined[2,5].

The clinical presentation of CD depends on the region of the bowel involved, the degree of inflammation, and the presence of complications. Children with ileocolitis typically present with crampy abdominal pain and diarrhea, which may be bloody. Systemic signs and symptoms such as fever, malaise, easy fatigability, and growth failure, are common in CD. Gastric and duodenal involvement may cause vomiting and epigastric pain. Perianal disease is common in CD. The clinical presentation of UC typically includes bloody diarrhea with mucus. More severe cases may also present with tenesmus, urgency, crampy abdominal pain, and nocturnal bowel movements. Onset is usually insidious with gradual progression of symptoms. Fever, severe anemia, hypoalbuminemia, and leukocytosis may also be present. Presentation may be milder in cases involving only the rectum. Systemic manifestations occur less commonly than in CD. UC is associated with an increased risk of colon cancer. Secondary amenorrhea is common during periods of active disease in both CD and UC[5,6].

Both CD and UC were identified as clinical disorders in the early 20th century. Population-based studies have suggested an uneven distribution of IBD throughout the world with the highest disease rates occurring in "Westernized" countries[6]. The reported incidence of CD is 3-4/100 000 and the prevalence is 30-100/100 000. Earlier age at onset is associated with more severe disease and with increased likelihood of CD in family members. Incidence rates for UC are highest in European countries and the United States (15/100 000) and lowest in Japan and South Africa (1/100 000). The incidence in Israel varies by country of origin with the lowest rates among those from Asia or Africa. The prevalence of UC in European countries and the United States is 100-200/100 000. In Europe and North America, the incidence of IBD has increased steadily since the first half of the 20th century[7,8]. In particular, the incidence of CD has increased although the incidence of UC has generally reached a plateau in the second half of the 20th century (Tables 2 and 3). Of note, the incidence of both CD and UC in children has been increasing.

Rapid changes in the incidence of IBD within the same population can best be explained by changes in environmental factors since changes in genetic predisposition do not occur rapidly[7]. Some studies have demonstrated a predilection of IBD for urban rather than rural populations or a north-south gradient of disease incidence[9]. The incidence of IBD in individuals of Jewish ancestry is higher than in individuals not of Jewish ancestry. Migrant studies have shown that immigrants acquire IBD at a rate consistent with that of the new geographic area[7]. This evidence suggests that there is a significant influence of environmental factors on the development of IBD.

A number of factors, including smoking, oral contraceptive (OC) agents and diet, have been considered as potential risk factors for IBD. Smoking, the most extensively studied environmental factor in the development of IBD, is a risk factor for CD[10], but a protective factor for UC[11]. A meta-analysis of smoking and IBD confirmed these findings (Table 4)[12]. The effect of passive smoke exposure in childhood on the subsequent development of IBD remains inconclusive (Table 4). The relationship between OC agents and the development of IBD is less certain. Epidemiologic investigations of the relationship between OC agents and IBD yielded mixed results. A meta-analysis of these studies showed evidence of a modestly increased risk for CD and UC in OC users (Table 4)[13]. A more recent study confirmed the modestly increased risk of CD and UC in former and current users of OC agents, but the difference was statistically significant only for current users of OC agents and CD [ψ = 3.4 (1.0-11.9), n = 106 age-matched pairs][14]. Because of the direct interface between diet and the gastrointestinal tract, the role of dietary factors in the development of IBD has been extensively investigated. Early studies of diet had serious methodological flaws and their findings have been questioned[15]. A Japanese study of 101 cases and 143 controls found an increased risk of UC associated with consumption of Western foods (P_{trend} = 0.04)[16]. More recently, a well-designed study of diet was conducted in newly diagnosed IBD patients [CD (n = 33), UC (n = 54)] (Table 4)[17]. A decreased risk of CD was associated with increasing consumption of vitamin C. An increased risk of UC was associated with increasing consumption of sucrose, animal fat, cholesterol, and soft drinks.

The role of preceding infections in the development of IBD is unclear (Table 4). In a matched case-control study, patients with CD had a higher rate of gastroenteritis in the first six months of life than controls, but patients with UC did not[18]. In separate studies, children with CD[19] and children with UC[20] were more likely than their unaffected siblings to have had diarrheal illness during infancy. In a large study

Table 2 Incidence of Crohn's disease

Annual incidence per 100 000	Time period	Geographic region	Data collection	Age group studied	References
0.73	1958-1960	Nottingham, England	Retrospective	Adults	[70]
3.63	1970-1972				[70]
6.1	1971-1980	Orebro, Sweden	Retrospective	Children ≤ 16 years old	[71]
[1]3.39 (white males)	1977-1979	Baltimore SMSA	Retrospective	All	[10]
[1]3.54 (white females)					[10]
[1]1.29 (non-white males)					[10]
[1]4.08 (non-white females)					[10]
0.66	1968	Scotland	Retrospective	Children ≤ 16 years old	[72]
2.29[b]	1983				[72]
0.00	1920-1929	Rochester, NY	Retrospective	All	[73]
5.03	1970-1979				[73]
3.90	1980-1989				[73]
< 1.0	1962-1969	Copenhagen	Prospective	All	[74]
4.1	1979-1987				[74]
1.30	1983-1988	South Glamorgan, Wales	Retrospective	Children < 16 years old	[75]
3.11	1989-1993				[75]
[2]1.0	1940-1943	Olmsted County, MN	Retrospective	All	[76]
[2]7.8	1964-1973				[76]
[2]6.9	1984-1993				[76]
14.6	1989-1994	Manitoba, Canada	Retrospective	All	[77]
[2]1.91	1981-1983	Scotland	Retrospective	Children < 19 years old	[78]
[2]2.91	1990-1992				[78]
5.5	1990-1994	Iceland	Prospective	All	[79]
1.2	1984-1986	Sweden	Prospective	Children < 16 years old	[80]
1.3	1993-1995				[80]
[2]5.2	1988-1990	Northern France	Prospective	All	[81]
[2]5.8	1991-1993				[81]
[2]5.9	1994-1996				[81]
[2]6.4	1997-1999				[81]
2.00	1990-1994	Southeastern Norway	Prospective	Children < 16 years old	[82]
4.56	2000-2001	Wisconsin	Prospective	Children < 18 years old	[83]

[1]Age-adjusted; [2]Age- and gender-adjusted; [b]$P < 0.0001$ compared to 1968.

(257 cases of IBD with 2 matched controls per case) of perinatal risk factors for IBD, the greatest risk was associated with postnatal infections in the child [ψ = 5.5 (2.6-11.8)][21]. In a nested case-control study (26 CD, 29 UC, eight randomly selected controls matched for gender and social class) from two national longitudinal birth cohorts, infections during pregnancy or in childhood were associated with an increased risk of CD and UC, but these differences were not statistically significant[22]. In a large, international multicenter study of children with IBD and sex and age-matched controls, recurrent respiratory infections were significantly more common in CD and UC patients than their controls and patients with CD used antibiotics more frequently than their controls[23]. In this study, there were no differences between cases and controls in the frequency of gastroenteritis severe enough to require hospitalization, the age of its occurrence, the frequency of other hospitalizations, other recurrent infections, or tonsillectomy/adenoidectomy[23]. In another study, adults with CD reported an increased frequency of childhood infections compared to neighbor controls as well as more frequent treatment with antibiotics for both otitis and pharyngitis[24]. In this same study, adults with UC reported more frequent childhood infections than neighbor controls, but no increased frequency of antibiotic treatment[24].

The role of perinatal and childhood factors in the development of IBD has been investigated, but very little has been demonstrated (Table 4). No significant differences were found in birth weight, prematurity, birth at home, nursery school attendance, number of children in nursery or first class at school, number of playmates, bedroom sharing with other children, home environment at different ages, birth month, or the number of siblings but children with CD were more likely than controls to be last born ($P < 0.02$). The age interval to the previous sibling did not differ between last-born patients and controls[23]. Two smaller studies found no association between seasonality of birth, maternal age at birth, birth weight, or birth order and either disease[25,26]. In a study of perinatal factors, the occurrence of any perinatal health event increased the risk of both CD and UC[21]. The occurrence of any noninfectious perinatal event was an independent risk factor for IBD [ψ = 3.5 (2.0-6.3)] as was low socioeconomic status [ψ = 2.7 (1.2-5.7)] and low placental weight [ψ = 1.5 (1.0-2.2)]. A large, population-based case-control study from Sweden used siblings as a marker for exposure pattern[27]. Cases were identified through the Swedish Inpatient Register and controls (matched by age and area of residence) through Swedish Census, Birth and Death registers. Analyses were adjusted for sex, multiple birth, maternal age, region, year of birth, and fathers' social class. There was a significant, graded negative association between CD and number of younger siblings. Increasing maternal age was

Table 3 Incidence of ulcerative colitis

Annual incidence per 100 000	Time period	Geographic region	Data collection	Age group studied	References
[1]2.92 (white males)	1977-1979	Baltimore SMSA	Retrospective	All	[10]
[1]1.79 (white females)					[10]
[1]1.29 (non-white males)					[10]
[1]2.90 (non-white females)					[10]
1.91	1968	Scotland	Retrospective	Children ≤ 16 years old	[72]
[3]1.56	1983				[72]
0.06	1920-1929	Rochester, NY	Retrospective	All	[73]
3.51	1970-1979				[73]
2.32	1980-1989				[73]
6.9	1962-1969	Copenhagen	Prospective	All	[84]
9.2	1980-1987				[84]
0.71	1983-1993	South Glamorgan, Wales	Retrospective	Children < 16 years old	[75]
14.3	1989-1994	Manitoba, Canada	Retrospective	All	[77]
16.5	1990-1994	Iceland	Prospective	All	[79]
1.4	1984-1986	Sweden	Prospective	Children < 16 years old	[80]
3.2[a]	1993-1995				[80]
[2]4.2	1988-1990	Northern France	Prospective	All	[81]
[2]4.3	1991-1993				[81]
[2]3.9	1994-1996				[81]
[2]3.5	1997-1999				[81]
2.14	1990-1994	Southeastern Norway	Prospective	Children < 16 years old	[77]
2.14	2000-2001	Wisconsin	Prospective	Children < 18 years old	[83]

[1]Age-adjusted; [2]Age- and gender-adjusted; [3]$P = 0.052$ compared to 1968; [a]$P < 0.05$ compared to 1984-1986.

negatively associated with CD ($P < 0.001$). There was a protective effect of younger siblings that was greatest for those born soon after subjects, with statistical significance disappearing for those born 5 years later than subjects. There was a significant, graded positive association between UC and number of older siblings. Maternal age was not consistently associated with UC. There was no discernible pattern in the relationship with UC risk by age difference between subjects and their older siblings[27].

Several studies have shown an increased risk of IBD in relatives of individuals with CD compared to the general population. Population studies found that first-degree relatives of CD patients had a prevalence of CD that was 10-21 times the population prevalence and a prevalence of UC that was 6-10 times the population prevalence[28,29]. Relatives of a patient with CD had a greater risk of acquiring CD than UC and relatives of a patient with UC had a greater risk of acquiring UC than CD, but both diseases could occur in the same family[28,30]. In general, the familial association has been greater for individuals with CD than for those with UC[28]. Twin studies have demonstrated greater concordance for CD than for UC and greater concordance for monozygotic twins than for dizygotic twins[31-33]. These observations suggest that there is an inherited predisposition to the development of CD and UC.

GENETIC FACTORS

Several groups of investigators have conducted studies to discern the modes of inheritance of CD and UC. In one study, segregation analysis of 265 CD patients and 5387 relatives suggested a recessive susceptibility gene for CD with incomplete penetrance. The model predicted that the proportion of cases explained by the presence of this gene would be very high among those with early onset disease and about 30% of cases would be due to homozygosity for the gene[34]. In a second study, complex segregation analysis of 133 CD patients and their relatives also suggested a recessive major locus, however, with nearly complete penetrance. This model also predicted that 7% of patients would be homozygous for the recessive gene, but 28% of patients under age 20 would be homozygous for the recessive gene[35]. In UC patients, segregation analysis of 65 patients and their relatives suggested a rare additive major gene that would account for 11% of the total phenotypic variance and would have penetrance for heterozygotes of 0.22. The model predicted that affected individuals would be more likely to be heterozygous than homozygous for the additive gene and risk to an offspring of an affected individual would be 11%[36].

Subsequent genome-wide scanning studies have identified a series of IBD susceptibility loci[37-39]. Some of these loci are more strongly associated with CD, others with UC, and some with both. With respect to CD, the most recent genome-wide scanning study confirmed previously established associations at IBD1, IBD5 (5q31), IL23R, ATG16L1, IRGM, TNFSF15, and PTPN2, but also identified 21 new loci associated with CD[40]. The first locus identified, IBD1, has shown evidence for linkage with CD, but not with UC[41]. Definitive evidence for linkage at IBD1 was confirmed in a large, international IBD genetics consortium study that also demonstrated equally increased allele sharing at this locus in Jewish and non-Jewish cohorts[42]. In 2001, three major, relatively uncommon, single nucleotide polymorphisms (SNPs) were identified at a gene in

Table 4 Factors affecting development of IBD

Factor	Effect	Findings
Cigarette smoking	Protective factor for UC	Pooled OR for UC = 0.41 (0.34-0.48); χ^2 = 11.52 (P < 0.001)[12]
	Risk factor for CD	Pooled OR for CD = 2.0 (1.65-2.47); χ^2 = 48.4 (P < 0.001)[12]
Passive cigarette smoke	Uncertain	No effect[25,85]
		UC [ψ = 0.50 (0.25-1.00), *n* = 163][86]
		CD [ψ = 5.32 (1.09-25.9), *n* = 39 age and sex-matched pairs][87]
		UC [ψ = 2.19 (0.75-6.41), *n* = 33 age and sex-matched pairs][87]
Oral contraceptive use	Risk factor for UC	Pooled RR for UC = 1.29 [(0.94-1.77) adjusted for smoking]
	Risk factor for CD	Pooled RR for UC = 1.68 [(0.97-2.88) unadjusted for smoking]
		Pooled RR for CD = 1.44 [(1.12-1.86) adjusted for smoking]
		Pooled RR for CD = 1.68 [(0.97-2.88) unadjusted for smoking]
Diet	Protective factor for CD	
	Vitamin C	ψ = 0.48 and ψ = 0.23 for medium and high intake, respectively, *vs* low intake, P_{trend} = 0.02[17]
	Risk factors for UC	
	Sucrose	[Sucrose] ψ = 2.05 and ψ = 4.22 for medium and high intake, respectively, *vs* low intake, P_{trend} = 0.02
	Animal fat	[Animal fat] ψ = 2.02 and ψ = 4.09 for medium and high intake, respectively, *vs* low intake, P_{trend} = 0.02
	Cholesterol	[Cholesterol] ψ = 2.14 and ψ = 4.57 for medium and high intake, respectively, *vs* low intake, P_{trend} = 0.02
	Soft drinks	[Soft drinks] ψ = 1.84 and ψ = 3.39 for medium and high intake, respectively, *vs* low intake, *vs* P_{trend} = 0.02[17]
Infections	Risk factor for CD/possible risk factor for UC	CD patients had a higher rate of gastroenteritis than did controls (6/57 *vs* 1/114, *P* = 0.005)[8]
		UC patients and controls did not differ (4/51 *vs* 1/102, *P* = NS)[8]
	Gastroenteritis	Children with CD were more likely than unaffected siblings to have had diarrheal illness [RR = 2.7 (95% CI 1.5-5.8) *P* < 0.02, *n* = 294] Children with UC were more likely than unaffected siblings to have had diarrheal illness [RR = 3.2 (95% CI 1.15-8.75), *P* = 0.03, *n* = 231][20]
	Diarrheal illness in infancy	
	Risk factor for CD and UC	Recurrent respiratory infections were significantly more common in CD patients and in UC patients than their controls (102/298 *vs* 156/601 and 73/194 *vs* 106/393, respectively, both *P* < 0.01)[7]
	Recurrent respiratory infections	Adults with CD had an increased frequency of childhood infections compared to neighbor controls [ψ = 4.67, (95% CI 2.65-8.23) *n* = 322 cases, 262 controls][24]
	Childhood infections	Adults with UC had more frequent childhood infections than neighbor controls [ψ = 2.37 (95% CI 1.19-4.71) (*n* = 181 cases, 141 controls)][24]
Antibiotic use	Risk factor for CD	Patients with CD used antibiotics more frequently than controls (*P* < 0.01)[7]
		Adults with CD had more frequent treatment with antibiotics for both otitis [ψ = 2.07 (95% CI 1.03-4.14)] and pharyngitis [ψ = 2.14 (95% CI 1.20-3.84)] than controls[24]
Perinatal factors	Risk factor for UC	For UC, the odds ratios for having one, two, and three or more older siblings were 1.08 (1.03-1.14), 1.09 (1.01-1.16), and 1.12 (1.02-1.23), respectively (*n* = 15 823 cases; 79 546 controls)[27]
	Number of older siblings	
	Protective factor for CD	For CD, the odds ratios for having one, two, and three or more younger siblings were 0.93 (0.88-0.99), 0.89 (0.82-0.96), and 0.83 (0.75-0.92), respectively (*n* = 12 668 cases; 63 035 controls)[27]
	Number of younger siblings	

this locus, the *NOD2/CARD15* gene that conferred susceptibility to CD[41,43]. One group identified three separate SNPs in the *NOD2* gene [a frameshift variant (L1007fsinsC) and two missense variants (R702W and G908R)] which were associated with CD. The genotype relative risks for CD in their sample compared to those with no mutations, for simple heterozygous individuals, homozygous individuals, and compound heterozygous individuals (i.e. those with two different variant alleles) were 3, 38, and 44, respectively. The demonstrated gene-dosage effect suggested a recessive model of inheritance. The other group identified only the frameshift variant SNP. The reported genotype relative risks for heterozygous and homozygous individuals were 1.5 and 17.6, respectively. A third group confirmed the presence of the frameshift variant in two different cohorts of patients[44]. For CD, the mutation was highly associated [heterozygotes and homozygotes *vs* normal, ψ = 2.6 (1.5-4.5), ψ = 42.1 (4.3-∞), respectively]. In all of these studies, the gene mutation was not associated with UC. Subsequent studies by different investigators in different Caucasian cohorts have confirmed that these NOD2 variants were independent risk factors for CD, conferring susceptibility for CD[45-49]. Overall, 27%-32% of CD patients carry one major variant allele compared to 10%-20% of Caucasian controls and 8%-17% of CD patients carry two major variant alleles compared to 1%-5% of Caucasian controls[35,50].

The *NOD2* gene encodes for a protein in monocytes that is involved in the immune-mediated inflammatory response to enteric pathogens. The frameshift variant truncates the NOD2 protein and is associated with a marked hyporesponsiveness of NF-κB activation with lipopolysaccharide treatment. The missense variants yield a NOD2 protein that showed a greater response to lipopolysaccharide, but still a diminished ability to activate NF-κB. How the mutant NOD2 proteins and impaired NF-κB activation confer susceptibility to CD is unknown. However, it is known that NOD2 protein plays a critical role in the detection of bacterial muramyl dipeptide, and can activate the adaptive immune system by acting as an adjuvant receptor for antibody production[37,51].

A number of investigators have attempted to define demographic and clinical features associated with the NOD2 variants known to be associated with CD. Some have identified a younger age of onset of CD associated with the NOD2 variants, particularly the frameshift variant and particularly for homozygotes or compound heterozygotes[45,47] while others have not[46,49,52,53]. No studies have reported any relationship between gender and the NOD2 variants. In one study, the frequency of NOD2 variant alleles was significantly higher in familial

cases of CD than in sporadic cases of CD [30.9% (n = 173) *vs* 19.3% (n = 405), $P < 0.001$][46], but in two other studies the frequency of NOD2 variant alleles did not differ between familial and sporadic cases[47,49]. The reported NOD2 variants confer risk primarily in Caucasians, since they were not found in Asians with CD[54], and were found in much lower frequencies in African-Americans with CD[55]. NOD2 variants have been associated with ileal (or ileocolonic) involvement and stricturing disease[45-47,49,52,56,57]. In most of these studies, homozygous and compound heterozygous patients had increased risk of ileum-specific disease[45], stricturing disease[47,52], or both. Of the known NOD2 variants, the frameshift variant has the strongest association with ileum-specific disease[45] and stricturing disease[52].

BREASTFEEDING

The relationship between breastfeeding in infancy and subsequent development of IBD was first evaluated in the early 1960's by investigators who had observed that some patients with UC demonstrated a striking clinical relationship based on inclusion or exclusion of dairy products from their diet[58]. They conducted a case-control study of 132 adults with UC and 129 controls matched for age and sex. Patients with UC were more likely than controls to never have been breast-fed (χ^2 = 7.42, $0.001 < P < 0.01$) and to have been breast-fed 14 d or less (χ^2 = 9.05, $0.001 < P < 0.005$). Another group of investigators conducted a similar but smaller study with controls matched by age and sex to each of 51 adults with UC and 57 adults with CD. They found that UC patients were more likely never to have been breast-fed than controls (15/51 *vs* 12/102, $P = 0.005$), but there were no differences between CD patients and controls (11/57 *vs* 22/114, P = NS)[18]. However, a population-based case-control study in Sweden demonstrated a significantly shorter duration of breastfeeding in CD patients than in controls matched for sex and age (4.59 mo *vs* 5.76 mo, $P < 0.01$, n = 308 pairs)[59].

One group of investigators conducted two separate studies comparing infant feeding practices among children with IBD and their unaffected siblings. Compared to their unaffected siblings (n = 180), CD patients (n = 114) were less likely to have been breastfed [RR = 3.6 (95% CI 1.4-9.0), $P < 0.01$] and more likely to have received formula food from birth [RR = 3.1 (95% CI 1.3-7.4), $P < 0.02$]. CD patients were younger than their unaffected siblings ($P < 0.01$) but did not differ in gender, birth order, birth month, premature delivery, type of milk used for bottle feeding, age at introduction of solid foods, and length of exclusive and total length of breastfeeding. Multivariate analysis showed that only lack of breastfeeding and diarrheal diseases during infancy were independently associated with later development of CD[19]. In the second study, lack of breastfeeding did not differ significantly between UC patients (n = 93) and unaffected siblings (n = 138) [RR = 1.7 (95% CI 0.77-3.65), $P = 0.19$]. Multivariate analysis showed that children with UC were more likely than their unaffected siblings to be female ($P = 0.01$). UC patients and their unaffected siblings did not differ in age, duration of exclusive breastfeeding, total duration of breastfeeding, age at introduction of solid foods, birth order, or premature delivery[20].

A clinic-based pediatric study of 68 CD patients, 39 UC patients and 202 controls, demonstrated a protective effect of breastfeeding on development of CD [breastfeeding $\leqslant$ 5, 6-11, $\geqslant$ 12 mo *vs* not breastfeeding ψ 0.7 (0.3-1.5), 0.6 (0.2-1.5), 0.1 (0.01-1.10), respectively ($P_{trend} = 0.04$)], and a tendency toward a protective effect of breastfeeding on development of UC [breastfeeding $\leqslant$ 5, 6-11, $\geqslant$ 12 mo *vs* not breastfeeding ψ 0.7 (0.3-1.6), 0.5 (0.2-1.5), 0.2 (0.03-2.20), respectively ($P_{trend} = 0.07$)][25]. Both associations were controlled for maternal smoking. An Italian multi-center study of incident cases (594 UC patients and 225 CD patients) and randomly selected age and gender matched controls (patients with acute disease not related to smoking, OC use, or immunological disorders) showed an increased risk of IBD in those who had not been breastfed compared to those who had [UC ψ = 1.5 (95% CI 1.1-2.1) n = 594 pairs; CD ψ = 1.9 (95% CI 1.1-3.3) n = 225 pairs]. An increased risk of IBD was detected in subjects who had not been breastfed (controlling for smoking status and OC use), but was statistically significant only in females [UC ψ = 2.2 (95% CI 1.2-3.6) n = 240 pairs; CD ψ = 2.5 (95% CI 1.0-4.9) n = 106 pairs][14]. A Japanese study identified incident cases of IBD in children under the age of 15 years from a national epidemiological survey conducted from 1978 to 1993[60]. Healthy controls were matched to cases by age, sex, and block of birth. Children with CD were significantly less likely to have been breastfed during the first 4 mo of life than were healthy children [ψ = 0.3 (95% CI 0.13-0.70) n = 42 cases, 126 controls]. Children with UC were significantly less likely to have been breastfed during the first 4 mo of life than were healthy children [ψ = 0.53 (95% CI 0.31-0.89) n = 133 cases, 266 controls].

Quite a few smaller studies have evaluated the relationship between breastfeeding and development of IBD. Several of these studies showed a trend toward a protective effect of breastfeeding on the development of IBD, but were too small to achieve statistical significance[16,22,26,61]. Three studies conducted as postal questionnaires all showed no association between breastfeeding and either CD or UC[24,62,63]. These postal questionnaire studies in which cases identified their own controls may have suffered from selection bias[24,62,63] and one of these studies had a very poor response rate thus creating a potential for non-respondent bias[63]. Two large studies failed to demonstrate any differences between cases and controls with respect to breastfeeding in infancy[21,23]. Breastfeeding data for one of these studies was limited to that which was obtained from the hospital chart at the time of the child's delivery thus creating potential for differential misclassification bias[21]. Many of these studies that did not demonstrate a protective effect of breastfeeding on the development of IBD did not characterize breastfeeding as exclusive or mixed and did

not report the duration of breastfeeding. Furthermore, many of these studies did not include potential confounders of the relationship between breastfeeding and IBD. These confounders include family history of IBD, cigarette smoking, OC use, preceding infections, antibiotic use, and various perinatal factors.

Recently, a meta-analysis of all these studies was conducted[64]. Studies were graded based on predefined guidelines. Criteria for the highest grade included recruitment of cases and controls by the investigators, confirmation of diagnosis by a physician, confirmation of breastfeeding information by subjects' mothers or other close relatives, and response rate of at least 80% for both cases and controls. Only four studies received the highest grade for CD[19,21,25,59] and four for UC[20,21,25,58]. Based on all studies of the relationship between breastfeeding and IBD, there was a protective effect of breastfeeding on both CD [ψ_{pooled} = 0.67 (95% CI 0.52-0.86) $P < 0.001$ (heterogeneity test)] and UC [ψ_{pooled} = 0.77 (95% CI 0.61-0.96) P = 0.004 (heterogeneity test)]. Based on the highest grade of studies, the effect of breastfeeding was even more pronounced for both CD [ψ_{pooled} = 0.45 (95% CI 0.26-0.79) P = 0.063 (heterogeneity test)] and UC [ψ_{pooled} = 0.56 (95% CI 0.38-0.81) P = 0.268 (heterogeneity test)][65]. The investigators concluded that their meta-analysis supported the hypothesis that breastfeeding is protective for both CD and UC and that the actual effect is probably greater than their analysis demonstrated due to nondifferential misclassification in some of the studies analyzed.

Subsequently, a population-based, pediatric matched case-control study of environmental risk factors and development of IBD was conducted in Northern France[66]. All IBD cases diagnosed between 1988 and 1997 who were under 17 years of age and resident in the study area at the time of diagnosis were recruited for the study. Randomly selected controls were matched to cases by age, sex, and living area. Subjects were interviewed by trained interviewers and answers were validated using the mandatory child health booklet. Controlling for maternal education level, breastfeeding was an independent risk factor for CD [ψ = 2.1 (95% CI 1.3-3.4) P = 0.003, n = 222 pairs] as were family history of IBD, history of eczema, and BCG vaccination[66]. Drinking tap water (*vs* bottled water or well water) was a protective factor for CD. Regarding the unexpected finding of breastfeeding as a risk factor for CD, the investigators speculated that this association might be the result of either delayed infections at weaning or environmental contamination of the breast milk in the highly industrialized region in which the study was conducted. In the same study, there was no association between breastfeeding and development of UC. Controlling for maternal education, risk factors for UC included family history of IBD, disease during pregnancy, and bedroom sharing, but appendectomy was a protective factor for UC[66].

After publication of this case-control study[66], which met the criteria for the highest grade, the meta-analysis was repeated[64]. Including this study, the protective effect of breastfeeding on the development of CD was diminished [ψ_{MH} = 0.62 (95% CI 0.27-1.43)], but the protective effect of breastfeeding on the development of UC was not altered significantly [ψ_{MH} = 0.62 (95% CI 0.43-0.91)]. More importantly, the inclusion of the most recent study resulted in a much higher heterogeneity for the CD studies ($P < 0.001$, chi-square heterogeneity test). The investigators offered several possible explanations for the surprising different results of the highest quality studies. These included differences in genetic characteristics of the studies' populations, subtypes of CD with different etiologies, and variations in the components of breast milk in the different regions studied[64].

SUMMARY

Despite extensive investigation, the etiology of IBD is still unknown. Clearly, a genetic predisposition to IBD exists[28-30]. Genome-wide scanning studies have identified a series of IBD susceptibility loci, some of which are more strongly associated with CD, others with UC, and some with both[38-40]. Three separate mutations in the *NOD2* gene have been identified that confer susceptibility to CD[41,43], but no specific mutations that confer susceptibility to UC have yet been identified. Despite the strong evidence of genetic predisposition to IBD, it is clear that environmental factors also influence the development of IBD. However, only cigarette smoking has a well established association with IBD. Paradoxically, cigarette smoking is a risk factor for CD, but a protective factor for UC[12]. OC use may also play a role in the etiology of CD although, obviously, only in women[13,14]. Numerous dietary components may play a role in the etiology of IBD although these associations are less certain[16,17]. Many investigators have identified associations between preceding infections and the development of IBD[13,18-20,23,24] and some have identified associations between antibiotic use and development of IBD[23,24]. Many perinatal factors have been studied, but no consistent findings have been reported[21-23,25-27]. Although there have been conflicting reports, meta-analysis of these reports indicates that breastfeeding is a protective factor for both CD and UC[65].

Human breast milk contains many substances that may influence growth and development as well as function of the gastrointestinal tract. Some of these factors may have age-dependent effects[67]. Furthermore, the composition of colonic flora differs between breastfed and bottle-fed infants[68]. IBD pathogenesis is presumed to be a complex interaction between genetic predisposition and inappropriate activation of the mucosal immune system driven by the presence of enteric flora and resulting in tissue injury[1-3]. Thus, it seems quite plausible that breastfeeding would have a protective effect on the development of IBD in genetically predisposed individuals, at least in childhood.

The preponderance of evidence suggests that breastfeeding is a protective factor for IBD, with a greater effect for CD than UC[14,18,19,22,25,58-60]. A meta-analysis of all available studies, taking into account the design of the studies, demonstrated this protective effect of breastfeeding on the development of IBD[65]. However,

this relationship has become more tenuous following the most recent study of the relationship between breastfeeding and IBD[64,66]. Why some studies showed a protective effect of breastfeeding, some showed no effect, and two showed that breastfeeding is a risk factor for IBD is unclear. Proposed explanations include differences in genetic characteristics of the populations studied, subtypes of CD with different etiologies, and variations in the components of breast milk in the different regions studied[64]. The heterogeneous findings may also result from differences in study design. Specifically, the heterogeneous findings may be due to the failure to control for genetic predisposition. Since IBD is thought to occur in genetically predisposed hosts, inclusion of subjects whose genetic predisposition is unknown may be inappropriate. Since estimates of the frequency of NOD2 variants in the Caucasian population range from 4% to 20%[37,43,44,69], inclusion of general population controls may confound results of the investigation.

To date, no studies of the relationship between breastfeeding and IBD have incorporated genetic predisposition into the study design. Two studies have been conducted using unaffected siblings of cases as controls[20,21]. The first of these studies found that children with CD were less likely to have been breastfed [RR = 3.6 (95% CI 1.4-9.0) $P < 0.01$] than their unaffected siblings[19]. This is the strongest association between breastfeeding and CD in any of the published studies. The second study found that children with UC were less likely to have been breastfed than their unaffected siblings but the difference was not statistically significant[20]. Genetic predisposition is important in the etiology of both CD and, to a lesser degree, UC. These two studies were completed long before the discovery of the NOD2 variants that confer susceptibility to CD[41,43] and no susceptibility genes for UC have yet been identified. Nevertheless, these two studies may better reflect the true relationship between breastfeeding in infancy and the subsequent development of IBD than any of the other published studies. To better elucidate the relationship between breastfeeding, or any environmental factor, and the development of IBD, future studies should be conducted in such a way that genetic susceptibility to IBD is considered. Specifically, future studies of the etiology of IBD should be designed such that both environmental factors and genetic factors are incorporated in the same study and gene-environment interaction should be assessed.

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REFERENCES

1 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434
2 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
3 **Shanahan F**. Crohn's disease. *Lancet* 2002; **359**: 62-69
4 Breastfeeding and the use of human milk. American Academy of Pediatrics. Work Group on Breastfeeding. *Pediatrics* 1997; **100**: 1035-1039
5 **Ulshen M**. Inflammatory bowel disease. In: Behrman RE, Kliegman RM, Jenson HB. Nelson Textbook of Pediatrics. Philadelphia: WB Saunders, 2000: 2414
6 **Lashner BA**. Epidemiology of inflammatory bowel disease. *Gastroenterol Clin North Am* 1995; **24**: 467-474
7 **Gilat T**, Langman MJ, Rozen P. Environmental factors in inflammatory bowel disease. *Front Gastrointert Res* 1986; **11**: 158-176
8 **Whelan G**. Epidemiology of inflammatory bowel disease. *Med Clin North Am* 1990; **74**: 1-12
9 **Sonnenberg A**, McCarty DJ, Jacobsen SJ. Geographic variation of inflammatory bowel disease within the United States. *Gastroenterology* 1991; **100**: 143-149
10 **Calkins BM**, Lilienfeld AM, Garland CF, Mendeloff AI. Trends in incidence rates of ulcerative colitis and Crohn's disease. *Dig Dis Sci* 1984; **29**: 913-920
11 **Harries AD**, Baird A, Rhodes J. Non-smoking: a feature of ulcerative colitis. *Br Med J* (Clin Res Ed) 1982; **284**: 706
12 **Calkins BM**. A meta-analysis of the role of smoking in inflammatory bowel disease. *Dig Dis Sci* 1989; **34**: 1841-1854
13 **Godet PG**, May GR, Sutherland LR. Meta-analysis of the role of oral contraceptive agents in inflammatory bowel disease. *Gut* 1995; **37**: 668-673
14 **Corrao G**, Tragnone A, Caprilli R, Trallori G, Papi C, Andreoli A, Di Paolo M, Riegler G, Rigo GP, Ferrau O, Mansi C, Ingrosso M, Valpiani D. Risk of inflammatory bowel disease attributable to smoking, oral contraception and breastfeeding in Italy: a nationwide case-control study. Cooperative Investigators of the Italian Group for the Study of the Colon and the Rectum (GISC). *Int J Epidemiol* 1998; **27**: 397-404
15 **Persson PG**, Ahlbom A, Hellers G. Crohn's disease and ulcerative colitis. A review of dietary studies with emphasis on methodologic aspects. *Scand J Gastroenterol* 1987; **22**: 385-389
16 Dietary and other risk factors of ulcerative colitis. A case-control study in Japan. Epidemiology Group of the Research Committee of Inflammatory Bowel Disease in Japan. *J Clin Gastroenterol* 1994; **19**: 166-171
17 **Reif S**, Klein I, Lubin F, Farbstein M, Hallak A, Gilat T. Pre-illness dietary factors in inflammatory bowel disease. *Gut* 1997; **40**: 754-760
18 **Whorwell PJ**, Holdstock G, Whorwell GM, Wright R. Bottle feeding, early gastroenteritis, and inflammatory bowel disease. *Br Med J* 1979; **1**: 382
19 **Koletzko S**, Sherman P, Corey M, Griffiths A, Smith C. Role of infant feeding practices in development of Crohn's disease in childhood. *BMJ* 1989; **298**: 1617-1618
20 **Koletzko S**, Griffiths A, Corey M, Smith C, Sherman P. Infant feeding practices and ulcerative colitis in childhood. *BMJ* 1991; **302**: 1580-1581
21 **Ekbom A**, Adami HO, Helmick CG, Jonzon A, Zack MM. Perinatal risk factors for inflammatory bowel disease: a case-control study. *Am J Epidemiol* 1990; **132**: 1111-1119
22 **Thompson NP**, Montgomery SM, Wadsworth ME, Pounder RE, Wakefield AJ. Early determinants of inflammatory bowel disease: use of two national longitudinal birth cohorts. *Eur J Gastroenterol Hepatol* 2000; **12**: 25-30
23 **Gilat T**, Hacohen D, Lilos P, Langman MJ. Childhood factors in ulcerative colitis and Crohn's disease. An international cooperative study. *Scand J Gastroenterol* 1987; **22**: 1009-1024
24 **Wurzelmann JI**, Lyles CM, Sandler RS. Childhood infections and the risk of inflammatory bowel disease. *Dig Dis Sci* 1994; **39**: 555-560
25 **Rigas A**, Rigas B, Glassman M, Yen YY, Lan SJ, Petridou

E, Hsieh CC, Trichopoulos D. Breast-feeding and maternal smoking in the etiology of Crohn's disease and ulcerative colitis in childhood. *Ann Epidemiol* 1993; **3**: 387-392
26 **Gruber M**, Marshall JR, Zielezny M, Lance P. A case-control study to examine the influence of maternal perinatal behaviors on the incidence of Crohn's disease. *Gastroenterol Nurs* 1996; **19**: 53-59
27 **Montgomery SM**, Lambe M, Wakefield AJ, Pounder RE, Ekbom A. Siblings and the risk of inflammatory bowel disease. *Scand J Gastroenterol* 2002; **37**: 1301-1308
28 **Monsen U**, Bernell O, Johansson C, Hellers G. Prevalence of inflammatory bowel disease among relatives of patients with Crohn's disease. *Scand J Gastroenterol* 1991; **26**: 302-306
29 **Orholm M**, Munkholm P, Langholz E, Nielsen OH, Sorensen TI, Binder V. Familial occurrence of inflammatory bowel disease. *N Engl J Med* 1991; **324**: 84-88
30 **Monsen U**, Brostrom O, Nordenvall B, Sorstad J, Hellers G. Prevalence of inflammatory bowel disease among relatives of patients with ulcerative colitis. *Scand J Gastroenterol* 1987; **22**: 214-218
31 **Subhani J**, Montgomery SM, Pounder RE, Wakefield AJ. Concordance rates of twins and siblings in inflammatory bowel disease. *Gut* 1998; **42**: A40
32 **Thompson NP**, Driscoll R, Pounder RE, Wakefield AJ. Genetics versus environment in inflammatory bowel disease: results of a British twin study. *BMJ* 1996; **312**: 95-96
33 **Tysk C**, Lindberg E, Jarnerot G, Floderus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* 1988; **29**: 990-996
34 **Kuster W**, Pascoe L, Purrmann J, Funk S, Majewski F. The genetics of Crohn disease: complex segregation analysis of a family study with 265 patients with Crohn disease and 5,387 relatives. *Am J Med Genet* 1989; **32**: 105-108
35 **Orholm M**, Iselius L, Sorensen TI, Munkholm P, Langholz E, Binder V. Investigation of inheritance of chronic inflammatory bowel diseases by complex segregation analysis. *BMJ* 1993; **306**: 20-24
36 **Monsen U**, Iselius L, Johansson C, Hellers G. Evidence for a major additive gene in ulcerative colitis. *Clin Genet* 1989; **36**: 411-414
37 **Bonen DK**, Cho JH. The genetics of inflammatory bowel disease. *Gastroenterology* 2003; **124**: 521-536
38 **Van Limbergen J**, Russell RK, Nimmo ER, Satsangi J. The genetics of inflammatory bowel disease. *Am J Gastroenterol* 2007; **102**: 2820-2831
39 **Cho JH**. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 2008; **8**: 458-466
40 **Barrett JC**, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M, Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* 2008; **40**: 955-962
41 **Hugot JP**, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001; **411**: 599-603
42 **Cavanaugh J**. International collaboration provides convincing linkage replication in complex disease through analysis of a large pooled data set: Crohn disease and chromosome 16. *Am J Hum Genet* 2001; **68**: 1165-1171
43 **Ogura Y**, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001; **411**: 603-606
44 **Hampe J**, Cuthbert A, Croucher PJ, Mirza MM, Mascheretti S, Fisher S, Frenzel H, King K, Hasselmeyer A, MacPherson AJ, Bridger S, van Deventer S, Forbes A, Nikolaus S, Lennard-Jones JE, Foelsch UR, Krawczak M, Lewis C, Schreiber S, Mathew CG. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet* 2001; **357**: 1925-1928
45 **Ahmad T**, Armuzzi A, Bunce M, Mulcahy-Hawes K, Marshall SE, Orchard TR, Crawshaw J, Large O, de Silva A, Cook JT, Barnardo M, Cullen S, Welsh KI, Jewell DP. The molecular classification of the clinical manifestations of Crohn's disease. *Gastroenterology* 2002; **122**: 854-866
46 **Cuthbert AP**, Fisher SA, Mirza MM, King K, Hampe J, Croucher PJ, Mascheretti S, Sanderson J, Forbes A, Mansfield J, Schreiber S, Lewis CM, Mathew CG. The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology* 2002; **122**: 867-874
47 **Lesage S**, Zouali H, Cezard JP, Colombel JF, Belaiche J, Almer S, Tysk C, O'Morain C, Gassull M, Binder V, Finkel Y, Modigliani R, Gower-Rousseau C, Macry J, Merlin F, Chamaillard M, Jannot AS, Thomas G, Hugot JP. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet* 2002; **70**: 845-857
48 **Vavassori P**, Borgiani P, D'Apice MR, De Negris F, Del Vecchio Blanco G, Monteleone I, Biancone L, Novelli G, Pallone E. 3020insC mutation within the NOD2 gene in Crohn's disease: frequency and association with clinical pattern in an Italian population. *Dig Liver Dis* 2002; **34**: 153
49 **Vermeire S**, Wild G, Kocher K, Cousineau J, Dufresne L, Bitton A, Langelier D, Pare P, Lapointe G, Cohen A, Daly MJ, Rioux JD. CARD15 genetic variation in a Quebec population: prevalence, genotype-phenotype relationship, and haplotype structure. *Am J Hum Genet* 2002; **71**: 74-83
50 **Biank V**, Broeckel U, Kugathasan S. Pediatric inflammatory bowel disease: clinical and molecular genetics. *Inflamm Bowel Dis* 2007; **13**: 1430-1438
51 **Kobayashi KS**, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 2005; **307**: 731-734
52 **Abreu MT**, Taylor KD, Lin YC, Hang T, Gaiennie J, Landers CJ, Vasiliauskas EA, Kam LY, Rojany M, Papadakis KA, Rotter JI, Targan SR, Yang H. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology* 2002; **123**: 679-688
53 **Achkar JP**, Cho J, Brzezinski A, Vogel D, Duerr R. Clinical manifestations of the NOD2 gene in familial Crohn's disease. *Am J Gastroenterol* 2002; **97**: S260-S261
54 **Inoue N**, Tamura K, Kinouchi Y, Fukuda Y, Takahashi S, Ogura Y, Inohara N, Nunez G, Kishi Y, Koike Y, Shimosegawa T, Shimoyama T, Hibi T. Lack of common NOD2 variants in Japanese patients with Crohn's disease. *Gastroenterology* 2002; **123**: 86-91
55 **Bonen DK**, Nicolae DL, Moran T, Turkyilmaz MA, Ramos R, Karaliukas R, Brant SR, Duerr RH, Kirschner B, Hanauer SB, Cho JH. Racial differences in NOD2 variation: characterization of NOD2 in African-Americans with Crohn's disease. *Gastroenterology* 2002; **122**: A-29
56 **Kugathasan S**, Collins N, Maresso K, Hoffmann RG, Stephens M, Werlin SL, Rudolph C, Broeckel U. CARD15

gene mutations and risk for early surgery in pediatric-onset Crohn's disease. *Clin Gastroenterol Hepatol* 2004; **2**: 1003-1009
57 **Sun L**, Roesler J, Rosen-Wolff A, Winkler U, Koch R, Thurigen A, Henker J. CARD15 genotype and phenotype analysis in 55 pediatric patients with Crohn disease from Saxony, Germany. *J Pediatr Gastroenterol Nutr* 2003; **37**: 492-497
58 **Acheson ED**, True Love SC. Early weaning in the aetiology of ulcerative colitis. A study of feeding in infancy in cases and controls. *Br Med J* 1961; **2**: 929-933
59 **Bergstrand O**, Hellers G. Breast-feeding during infancy in patients who later develop Crohn's disease. *Scand J Gastroenterol* 1983; **18**: 903-906
60 **Urashima H**, Ohmori I, Shiraki K. Epidemiological survey on chronic inflammatory bowel disease developed during childhood in Japan, and a case-control study on nutrition during infancy. *Yonago Acta Medica* 1999; **42**: 95-102
61 **Klein I**, Reif S, Farbstein H, Halak A, Gilat T. Preillness non dietary factors and habits in inflammatory bowel disease. *Ital J Gastroenterol Hepatol* 1998; **30**: 247-251
62 **Persson PG**, Leijonmarck CE, Bernell O, Hellers G, Ahlbom A. Risk indicators for inflammatory bowel disease. *Int J Epidemiol* 1993; **22**: 268-272
63 **Thompson NP**, Pounder RE, Wakefield AJ. Perinatal and childhood risk factors for inflammatory bowel disease: a case-control study. *Eur J Gastroenterol Hepatol* 1995; **7**: 385-390
64 **Klement E**, Reif S. Breastfeeding and risk of inflammatory bowel disease. *Am J Clin Nutr* 2005; **82**: 486
65 **Klement E**, Cohen RV, Boxman J, Joseph A, Reif S. Breastfeeding and risk of inflammatory bowel disease: a systematic review with meta-analysis. *Am J Clin Nutr* 2004; **80**: 1342-1352
66 **Baron S**, Turck D, Leplat C, Merle V, Gower-Rousseau C, Marti R, Yzet T, Lerebours E, Dupas JL, Debeugny S, Salomez JL, Cortot A, Colombel JF. Environmental risk factors in paediatric inflammatory bowel diseases: a population based case control study. *Gut* 2005; **54**: 357-363
67 **Carver JD**, Barness LA. Trophic factors for the gastrointestinal tract. *Clin Perinatol* 1996; **23**: 265-285
68 **Rubaltelli FF**, Biadaioli R, Pecile P, Nicoletti P. Intestinal flora in breast- and bottle-fed infants. *J Perinat Med* 1998; **26**: 186-191
69 **Hugot JP**, Laurent-Puig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L, Naom I, Dupas JL, Van Gossum A, Orholm M, Bonaiti-Pellie C, Weissenbach J, Mathew CG, Lennard-Jones JE, Cortot A, Colombel JF, Thomas G. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature* 1996; **379**: 821-823
70 **Miller DS**, Keighley AC, Langman MJ. Changing patterns in epidemiology of Crohn's disease. *Lancet* 1974; **2**: 691-693
71 **Lindquist BL**, Jarnerot G, Wickbom G. Clinical and epidemiological aspects of Crohn's disease in children and adolescents. *Scand J Gastroenterol* 1984; **19**: 502-506
72 **Barton JR**, Gillon S, Ferguson A. Incidence of inflammatory bowel disease in Scottish children between 1968 and 1983; marginal fall in ulcerative colitis, three-fold rise in Crohn's disease. *Gut* 1989; **30**: 618-622
73 **Stowe SP**, Redmond SR, Stormont JM, Shah AN, Chessin LN, Segal HL, Chey WY. An epidemiologic study of inflammatory bowel disease in Rochester, New York. Hospital incidence. *Gastroenterology* 1990; **98**: 104-110
74 **Munkholm P**, Langholz E, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of Crohn's disease in the county of Copenhagen, 1962-87: a sixfold increase in incidence. *Scand J Gastroenterol* 1992; **27**: 609-614
75 **Cosgrove M**, Al-Atia RF, Jenkins HR. The epidemiology of paediatric inflammatory bowel disease. *Arch Dis Child* 1996; **74**: 460-461
76 **Loftus EV Jr**, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Crohn's disease in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gastroenterology* 1998; **114**: 1161-1168
77 **Bernstein CN**, Blanchard JF, Rawsthorne P, Wajda A. Epidemiology of Crohn's disease and ulcerative colitis in a central Canadian province: a population-based study. *Am J Epidemiol* 1999; **149**: 916-924
78 **Armitage E**, Drummond H, Ghosh S, Ferguson A. Incidence of juvenile-onset Crohn's disease in Scotland. *Lancet* 1999; **353**: 1496-1497
79 **Bjornsson S**, Johannsson JH. Inflammatory bowel disease in Iceland, 1990-1994: a prospective, nationwide, epidemiological study. *Eur J Gastroenterol Hepatol* 2000; **12**: 31-38
80 **Lindberg E**, Lindquist B, Holmquist L, Hildebrand H. Inflammatory bowel disease in children and adolescents in Sweden, 1984-1995. *J Pediatr Gastroenterol Nutr* 2000; **30**: 259-264
81 **Molinie F**, Gower-Rousseau C, Yzet T, Merle V, Grandbastien B, Marti R, Lerebours E, Dupas JL, Colombel JF, Salomez JL, Cortot A. Opposite evolution in incidence of Crohn's disease and ulcerative colitis in Northern France (1988-1999). *Gut* 2004; **53**: 843-848
82 **Bentsen BS**, Moum B, Ekbom A. Incidence of inflammatory bowel disease in children in southeastern Norway: a prospective population-based study 1990-94. *Scand J Gastroenterol* 2002; **37**: 540-545
83 **Kugathasan S**, Judd RH, Hoffmann RG, Heikenen J, Telega G, Khan F, Weisdorf-Schindele S, San Pablo W Jr, Perrault J, Park R, Yaffe M, Brown C, Rivera-Bennett MT, Halabi I, Martinez A, Blank E, Werlin SL, Rudolph CD, Binion DG. Epidemiologic and clinical characteristics of children with newly diagnosed inflammatory bowel disease in Wisconsin: a statewide population-based study. *J Pediatr* 2003; **143**: 525-531
84 **Langholz E**, Munkholm P, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of ulcerative colitis in Copenhagen county from 1962 to 1987. *Scand J Gastroenterol* 1991; **26**: 1247-1256
85 **Persson PG**, Ahlbom A, Hellers G. Inflammatory bowel disease and tobacco smoke--a case-control study. *Gut* 1990; **31**: 1377-1381
86 **Sandler RS**, Sandler DP, McDonnell CW, Wurzelmann JI. Childhood exposure to environmental tobacco smoke and the risk of ulcerative colitis. *Am J Epidemiol* 1992; **135**: 603-608
87 **Lashner BA**, Shaheen NJ, Hanauer SB, Kirschner BS. Passive smoking is associated with an increased risk of developing inflammatory bowel disease in children. *Am J Gastroenterol* 1993; **88**: 356-359

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REVIEW

Liver cirrhosis and diabetes: Risk factors, pathophysiology, clinical implications and management

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Abstract

About 30% of patients with cirrhosis have diabetes mellitus (DM). Nowadays, it is a matter for debate whether type 2 DM in the absence of obesity and hypertriglyceridemia may be a risk factor for chronic liver disease. DM, which develops as a complication of cirrhosis, is known as "hepatogenous diabetes". Insulin resistance in muscular and adipose tissues and hyperinsulinemia seem to be the pathophysiologic bases of diabetes in liver disease. An impaired response of the islet β-cells of the pancreas and hepatic insulin resistance are also contributory factors. Non-alcoholic fatty liver disease, alcoholic cirrhosis, chronic hepatitis C (CHC) and hemochromatosis are more frequently associated with DM. Insulin resistance increases the failure of the response to treatment in patients with CHC and enhances progression of fibrosis. DM in cirrhotic patients may be subclinical. Hepatogenous diabetes is clinically different from that of type 2 DM, since it is less frequently associated with microangiopathy and patients more frequently suffer complications of cirrhosis. DM increases the mortality of cirrhotic patients. Treatment of the diabetes is complex due to liver damage and hepatotoxicity of oral hypoglycemic drugs. This manuscript will review evidence that exists in relation to: type 2 DM alone or as part of the metabolic syndrome in the development of liver disease; factors involved in the genesis of hepatogenous diabetes; the impact of DM on the clinical outcome of liver disease; the management of DM in cirrhotic patients and the role of DM as a risk factor for the occurrence and exacerbation of hepatocellular carcinoma.

INTRODUCTION

Up to 96% of patients with cirrhosis may be glucose intolerant and 30% may be clinically diabetic[1]. Currently, it is a matter for debate whether type 2 diabetes mellitus (DM), in the absence of other risk factors contributing to metabolic syndrome (obesity and hypertriglyceridemia), could be a risk factor for the development and progression of liver disease[2-4]. On the other hand, the diabetes which develops as a complication of cirrhosis is known as "hepatogenous diabetes" and is not recognized by the American Diabetes Association and the World Health Organization as a specific independent entity[5].

The liver has an important role in carbohydrate metabolism since it is responsible for the balance of blood glucose levels by means of glycogenogenesis and glycogenolysis[5-11]. In the presence of hepatic disease, the metabolic homeostasis of glucose is impaired as a result of disorders such as insulin resistance, glucose intolerance and diabetes[6,8,11,12]. Insulin resistance occurs not only in muscular tissue, but also in adipose tissue[13], and this combined with hyperinsulinemia seem to be important pathophysiologic bases of diabetes in liver disease[1,3,5,6,14-17]. Additionally, the etiology of liver disease is important in the incidence of DM, since non-alcoholic fatty liver disease (NAFLD), alcohol, hepatitis C virus (HCV) and hemochromatosis are frequently associated with DM[1-3,7,18].

DM in patients with compensated liver cirrhosis may be subclinical, since fasting serum glucose levels may be normal. In these cases, it is necessary to perform an oral

glucose tolerance test (OGTT) to detect an impairment of glucose metabolism[19]. The natural history of hepatogenous diabetes is different from that of hereditary type 2 DM, since it is less frequently associated with microangiopathy. In contrast, the patient with cirrhosis and diabetes suffers more frequently from complications of cirrhosis, which can cause death[2,4,19].

Treatment of diabetes in the cirrhotic patient is complex because of the presence of liver damage and the hepatotoxicity of oral hypoglycemic drugs. Therefore, pharmacological therapy must be closely monitored for the risk of hypoglycemia[3,5,19].

This review will present evidence that exists in the literature in relation to: (1) type 2 DM alone or as part of the metabolic syndrome in the development of liver disease; (2) factors involved in the genesis of hepatogenous diabetes; (3) the impact of DM on the clinical outcome of liver disease; (4) the management of DM in cirrhotic patients. Similarly, we will review the role of type 2 DM and hepatogenous diabetes as risk factors for the occurrence and exacerbation of hepatocellular carcinoma (HCC).

TYPE 2 DM AS A RISK FACTOR FOR NAFLD AND HCC

Epidemiology

Several studies suggest that type 2 DM may have an etiological role in chronic liver disease and HCC regardless of alcohol and viruses[4] (Figure 1).

A total of 173 643 patients with type 2 DM and 650 620 patients without type 2 DM, in whom chronic liver disease was excluded at the time of enrollment and a year later, were observed over a 10-year period in a cohort study. The incidence of non-alcoholic chronic liver disease and HCC was significantly higher in diabetic patients compared with non-diabetic patients. This risk was 2-fold greater and was independent of alcoholic liver disease, viral hepatitis and demographic factors[4]. Although this study has the strength of having a very large number of individuals, it has been criticized because it included a population comprised almost entirely of men (98%) from the Department of Veterans Affairs, and the diagnoses of type 2 DM, chronic liver disease and HCC were taken from a database, and consequently they were not verified biochemically and histopathologically. Additionally, other factors that are part of the metabolic syndrome (which already have a proven influence on the occurrence of NAFLD such as obesity and dyslipidemia) were not taken into account[20].

In a study with a large number of patients carried out in Denmark, the standardized incidence of HCC was higher in men (4.0, 95% CI: 3.5-4.6) and women (2.1, 95% CI: 1.6-2.7) with type 2 DM compared with the general population[21]. Other studies with fewer patients have yielded similar results[22,23].

In a recent case-control study that included 465 patients, DM prevalence was higher in patients with HCC than in controls (31.2% *vs* 12.7%, OR 3.12 95% CI: 2.22-4.43). The DM had been diagnosed prior to the occurrence of HCC in 84% of cases with an average duration of 181.4 mo indicating that it was type 2 DM in most cases[24]. The above data suggests that type 2 DM itself might be a risk factor for the occurrence of HCC. Other studies showed that in the presence HCV, liver fibrosis and alcohol, the risk is higher. Recently, it was observed that patients suffering from chronic hepatitis C (CHC), DM and advanced fibrosis had a 3-fold greater risk than non-diabetic patients with mild to moderate fibrosis of developing HCC in 5 years of follow-up (13% *vs* 5%)[25].

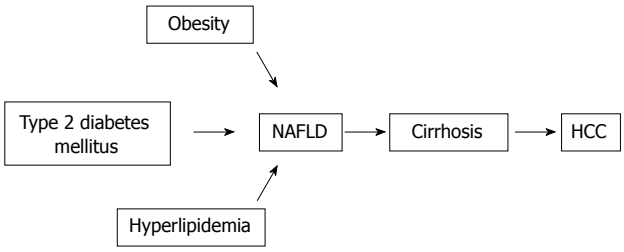

Figure 1 Type 2 diabetes mellitus may give raise to non-alcoholic fatty liver disease (NAFLD) which could progress to cirrhosis and hepatocellular carcinoma (HCC).

NAFLD

NAFLD comprises a series of liver disorders such as simple steatosis, steatohepatitis, fibrosis and cirrhosis. It is estimated that one third of American adult individuals may suffer from fatty liver[26], which is considered the most benign manifestation of NAFLD. The primary fatty liver results from the accumulation of fat, mainly of triglycerides in liver cells in the presence of insulin resistance, and frequently occurs as part of the metabolic syndrome which is made up of obesity, type 2 DM and dyslipidemia[27]. Non-alcoholic steatohepatitis (NASH) is a severe manifestation of NAFLD, since it causes not only steatosis, but tissue inflammation, cell damage and fibrosis. Nevertheless, the prevalence of NASH is estimated at 2%-3%. NASH is regarded as an entity that can progress to cirrhosis and liver failure, and currently it is estimated to be the most common cause of cryptogenic cirrhosis[28,29].

Pathophysiology

The mechanisms by which type 2 DM might cause NAFLD are complex and have been studied in a fragmented manner mainly in isolated biological systems.

It has been observed that the fatty liver, obesity and insulin resistance act as co-factors to cause liver damage[1,3,4]. Fatty liver is the result of an intracellular accumulation of triglycerides because of increased uptake of free fatty acids and *de novo* liponeogenesis in the hepatocytes. At the same time, there is a reduction in the hepatic secretion of very low density lipoproteins. The liver damage consists of cellular necrosis and inflammation, and these disorders result from an increase in mitochondrial oxidative stress on triglycerides with the consequential generation of free radicals and peroxisomes[30,31]. The mitochondrial oxidative

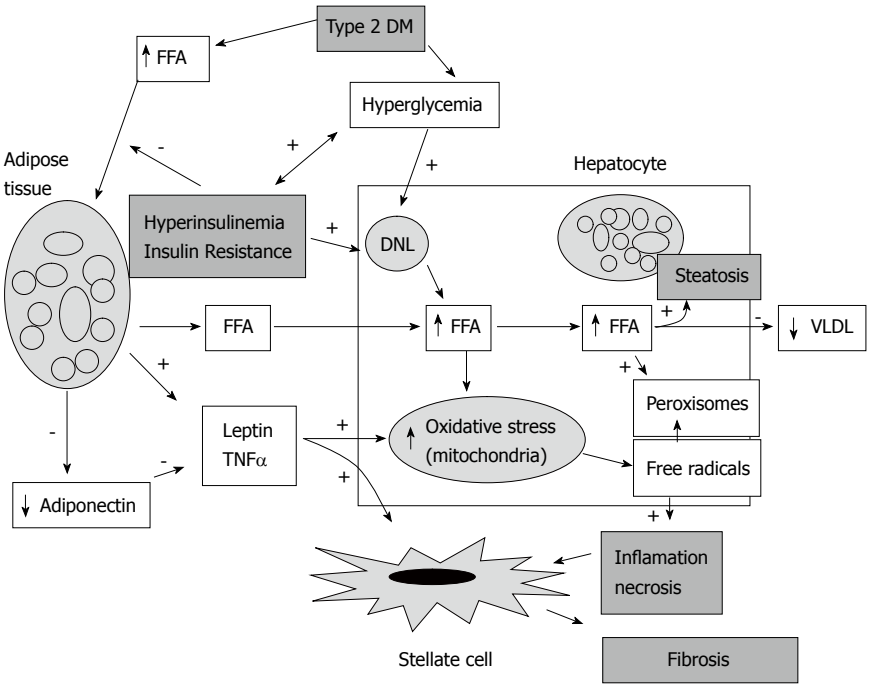

Figure 2 Liver damage caused by type 2 DM. Insulin resistance promotes release of free fatty acids (FFA) from adipose tissue. The FFAs are accumulated in the liver cells, and *de novo* liponeogenesis (DNL) contributes also. The reduced secretion of very low density lipoprotein (VLDL) by hepatic cells saturates hepatocytes producing steatosis. Mitochondrial oxidative stress is increased as a result of excess intracellular FFAs and the influence of adipokines (leptin and tumor necrosis factor alpha (TNF-α). Excess of oxidative stress produces free radicals which in turn induces inflammation and cellular necrosis. Tissue inflammation stimulates the stellate cells to produce collagen.

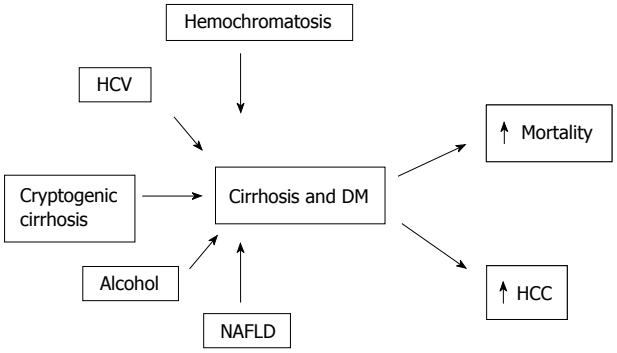

Figure 3 Etiology of liver cirrhosis most frequently associated with diabetes mellitus.

stress is increased also by the action of adipokines (cytokines produced by the adipocytes) such as leptin and tumor necrosis factor-α (TNF-α), which are produced in excess[32]. The reduction of adiponectin, which is a regulatory adipokine, favors the activity of inflammatory adipokines[33]. These chemical mediators, derived from inflammation and cell necrosis, as well as the adipokines activate the liver stellate cells and induce them to increase production of collagen, connective tissue growth factor and accumulation of extracellular matrix, in turn favoring fibrosis[34] (Figure 2).

DM AS A COMPLICATION OF CIRRHOSIS-HEPATOGENOUS DIABETES

Epidemiology

Depending on the etiology, the degree of liver damage and the diagnostic criteria, the reported incidence of glucose intolerance varies from 60 to 80% and that of diabetes between 20 and 60%[3,5,16,19]. It is known that from the early stages of chronic liver disease, insulin resistance and glucose intolerance may be found in most of these patients[35,36]. The diabetes manifests clinically as the liver function deteriorates, thus hepatogenous diabetes can be considered as an indicator of advanced liver disease[37].

The etiology of chronic liver disease is crucial in the development of hepatogenous diabetes: alcohol, HCV, hemochromatosis and NASH (Figure 3).

NASH: NASH is a severe manifestation of NAFLD. NASH is associated with visceral obesity, hypertriglyceridemia, and virtually all patients have insulin resistance. Therefore, it is not surprising that type 2 DM is present in 30%-45% of patients with NASH[38].

On the other hand, it has been observed that obesity itself is an independent risk factor for severe liver disease[39]. Obesity is characterized by expanded adipose tissue which is in a state of chronic inflammation resulting in an increase in the secretion of adipokines. These adipose tissue cytokines have a systemic effect particularly on the liver, which leads to an altered metabolic state with insulin resistance, hyperglycemia and hyperinsulinemia; these abnormalities disrupt the liver metabolism of lipids[40]. Cytokines, of which TNF-α is the most studied member, stimulate the liver stellate cells directly inducing hepatic fibrosis[41]. The body weight reduction improves metabolic abnormalities that accompany the metabolic syndrome such as hyperlipidemia and fatty liver[42].

CHC and HCV: In a study conducted by The National Health and Nutrition Examination Survey, a 3-fold higher risk of DM was identified in individuals over 40 years of age with CHC, compared with those patients with non-C chronic hepatitis[43]. Knobler *et al* observed a prevalence of DM of 33% in non-cirrhotic patients with CHC, compared with 5.6% in a control group[44]. In patients chronically infected with HCV, fatty liver was observed in 30%-70% of cases[45].

In patients with CHC, a high prevalence of glycometabolic abnormalities is reported such as glucose intolerance in more than 40% and DM in more than 17%. Additionally, the insulin resistance observed in these patients is an independent risk factor for steatosis in relation to the severity of fibrosis[7,46-48].

The mechanisms by which HCV produces insulin resistance and DM are not clearly known. It has been observed that HCV induces insulin resistance regardless of body mass index and fibrosis stage. In a study conducted in a transgenic animal model, the HCV core protein was able to induce insulin resistance, steatosis and DM. TNF-α overproduction seems to have been the primary mechanism. This cytokine phosphorylates the serine residues of the insulin receptor (IRS-1 and IRS-2), and stimulates the overproduction of suppressor of cytokines (SOC-3). SOC-3 inhibits phosphorylation of Akt and phosphatidylinositol 3-kinase. All these disorders, related to intracellular signaling of insulin, could block the transactivation of GLUT-4, which would result in block of glucose uptake at the cellular level. Indeed, in the transgenic mouse, TNF-α correlates with the hyperinsulinism and TNF-α block occasioned by the administration of anti-TNF-α drugs such as infliximab avoids the appearance of insulin resistance. Therefore, the mechanisms by which HCV induces insulin resistance include: production of TNF-α, serine phosphorylation of IRS and overexpression of SOCs. Furthermore, the overproduction of TNF-α in patients with CHC correlates with a faster progression of fibrosis and a lower response to interferon[40].

On the other hand, HCV genotype may be of importance in the occurrence of glucose metabolic disorders, as genotypes 1 and 4 are significantly associated with insulin resistance more frequently than genotypes 2 and 3 (37% *vs* 17%)[47]. It is well demonstrated that genotypes 1 and 4 are associated with a lower viral sustained response to antiviral therapy than genotypes 2 and 3. Insulin resistance may be a cofactor that increases the failure of the response to antiviral treatment observed in these patients. In accordance with this, in a recent study in patients with HCV genotype 1, those with HOMA > 2 (insulin resistance) had a 2-fold lower sustained response to treatment than patients with HOMA < 2 (32.8% *vs* 60.5%, respectively)[49]. For sustaining this idea, it is important to note that in experiments carried out with Huh-7 cells infected with HCV RNA, viral replication was blocked by adding interferon to the system. However, the ability of interferon to block viral replication was abolished when insulin was added to interferon at a dose of 128 mcU/mL (similar to that seen in the hyperinsulinemic states)[50]. Finally, it has been reported that patients with CHC and insulin resistance have a less sustained response to peginterferon plus ribavirin treatment compared with patients without insulin resistance[40,49].

It seems that once the insulin resistance and DM-inducing mechanisms in CHC are fired, their courses are not affected by the presence or absence of viral activity. Indeed, in a recent study it was observed that HCV clearance by pegylated interferon and ribavirin treatment did not reduce the risk of DM in patients with chronic hepatitis and normal fasting blood glucose during a period of 8 years of follow-up after treatment. Patients with a sustained response had a similar incidence of DM compared with those who did not respond to treatment (14.8% *vs* 18.5%, respectively)[51].

Alcohol: Patients with alcoholic liver disease have a high relative risk of suffering diabetes[52]. This risk is directly related to the amount of ingested alcohol, as it rises 2-fold in patients ingesting more than 270 g of alcohol per week compared with those ingesting less than 120 g/wk[53]. Acute alcohol ingestion produces a significant reduction in insulin-mediated glucose uptake. On the other hand, patients with chronic alcoholism frequently have chronic pancreatic damage and injury of pancreatic islet β-cells resulting in DM[1].

Hemochromatosis: Hereditary hemochromatosis is a disease characterized by iron accumulation in several organs-particularly in the liver - as a result of a disorder of the metabolism of this metal. This abnormality is produced by a mutation of the *HFE* gene. In addition, the iron can infiltrate the pancreas and myocardium. In the pancreas, the concentration of iron is predominantly in the acinus of exocrine secretion. However, infiltration of Langerhans islets with damage to the insulin-producing β-cells can also be observed. This is the reason why DM can be observed in 50%-85% of patients with hereditary hemochromatosis in advanced stages[54]. Additionally, glucose metabolic disorders resulting from the liver damage probably contribute to the high frequency of DM[1,5].

Pathophysiology of hepatogenous diabetes

The pathophysiology of hepatogenous diabetes is complex and not precisely known. Insulin resistance in peripheral tissues (adipose and muscular tissue) plays a central role in the glucose metabolism disturbance[1,2,9,11,14-17]. It has also been proposed that reduced insulin extraction by the damaged liver and portosystemic shunts result in hyperinsulinemia which is potentiated by raised levels of contra-insulin hormones (glucagon, growth hormone, insulin-like growth factor, free fatty acids and cytokines)[2,11,15,17]. However, a recent study reports that in patients with Child B grade liver cirrhosis the hyperinsulinism may be produced by an increase of the pancreatic β-cell sensitivity to glucose, whereas disturbance of hepatic insulin extraction does not seem to have a significant role[55]. It has also been speculated that genetic and environmental factors and some etiologic agents in liver disease such as HCV, alcohol, and iron infiltration impair the insulin secretion activity of the β-cells of the pancreas[9]. In conclusion it seems that glucose intolerance may result from two abnormalities that occur simultaneously: (1) insulin resistance of muscle and (2) an inadequate response of the β-cells to appropriately secrete insulin to overcome the defect in insulin action. On the other hand DM develops as the result of progressive impairment in insulin secretion together with the development of he-

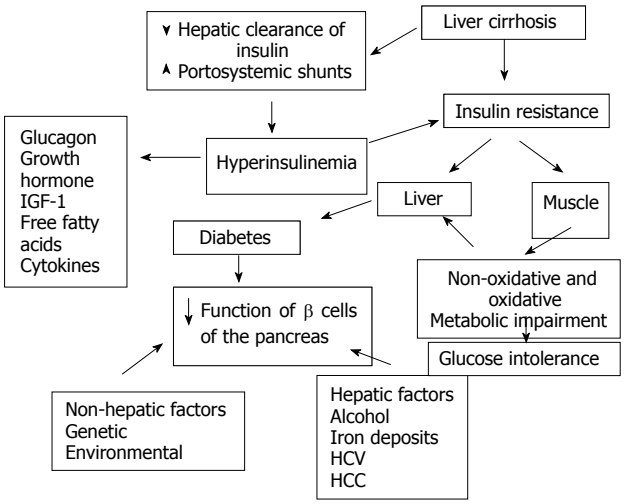

Figure 4 Pathophysiology of hepatogenous diabetes. One of the main abnormalities is insulin resistance in muscular cells and the hepatic tissue. Insulin resistance in muscle impairs non-oxidative and oxidative glucose metabolism. The reduction of insulin clearance by the damaged liver and the presence of portosystemic shunts in one hand and the desensitization of the beta cells of the pancreas produced by diverse factors on the other hand may produce hyperinsulinemia. With progression of the diabetes there is a reduction in sensitivity of β-cells for production of insulin.

patic insulin resistance leading to fasting hyperglycemia and a diabetic glucose tolerance profile[14,15] (Figure 4).

Discrimination between hepatogenous diabetes and type 2 DM may be difficult. In a recent study comparing patients with hepatogenous diabetes *vs* patients with type 2 DM, the ratios of postprandial plasma glucose (PP2h) to fasting plasma glucose (FPG) (2.27 *vs* 1.69), fasting insulin (23.2 *vs* 11.6 microIU/mL) and HOMA-Insulin Resistance index (8.38 *vs* 3.52) were significantly higher in patients with hepatogenous diabetes. Therefore, insulin resistance in liver cirrhosis is higher than in type 2 DM, and impairment of hepatic insulin degradation may be an important mechanism of hyperinsulinemia in liver cirrhosis[56].

TYPE 2 DM AND HEPATOGENOUS DIABETES AGGRAVATE LIVER CIRRHOSIS AND HCC

DM increases morbidity and mortality of liver cirrhosis patients

The effect of type 2 DM and hepatogenous diabetes on the clinical outcome of cirrhosis and HCC has been evaluated in only few studies. In cross-sectional retrospective studies in patients with cirrhosis of any etiology it has been observed that the DM is associated with an increased risk of complications[5,38,57,58]. According to the Verona study, which is based on a population of more than 7000 individuals suffering from type 2 DM, the risk of death at 5 years was 2.52-fold greater (CI 1.96-3.2) than in the general population[59]. Other studies report that DM, obesity and steatosis are associated with liver disease and more severe fibrosis in CHC[60,61].

It is important to note that neither the Child-Pugh nor Model for End-Stage Liver Disease (MELD) Scores (which are widely used as prognostic instruments of morbidity and mortality in the short and long term for cirrhotic patients) include in their parameters DM or glucose intolerance[62,63]. Nevertheless, interesting data have been observed in some prospective longitudinal studies involving cirrhotic patients where DM has been studied as an independent prognostic factor. In a retrospective and prospective study 354 (98 with diabetes) of 382 eligible patients were followed for 6 years after inclusion into the study: 110 were alive at the end of follow-up. Prognostic factors identified by Kaplan-Meier analysis, followed by Cox's stepwise regression demonstrated in sequence, albumin, ascites, age, encephalopathy, bilirubin, diabetes, and platelets as prognostic factors of mortality. The larger mortality rate in patients with diabetes, was not due to complications of diabetes but to an increased risk of hepatocellular failure[58]. Diabetes was no longer a risk factor as a covariate in a subgroup of 271 patients when varices were added but was again significant when patients who died of gastrointestinal bleeding were excluded.

In another study carried out in patients suffering from cirrhosis and refractory ascites on the waiting list for liver transplantation it was observed that the HCC and DM, but not the Child-Pugh score, were independent predictive factors of mortality. The patients suffering from refractory ascites and DM showed a 1- and 2-year probability of survival of 32% and 18%, respectively. By contrast survival rates of patients with refractory ascites without DM were 62% and 58%, respectively[64].

Nishida *et al* performed the OGTT on a group of 56 patients with cirrhosis and normal fasting blood glucose. A total of 38% of patients were diagnosed with DM, 23% with glucose intolerance, and 39% were normal. After 5 years of follow-up, patients with diabetes and glucose intolerance had significantly higher mortality than normal patients (44% and 32% *vs* 5%, respectively). From a multiple regression analysis only serum albumin and DM were independent negative predictive factors of survival[19].

Hepatogenous diabetes has a clinical behavior different from that of hereditary type 2 DM, since it is less frequently associated with retinopathy and cardiovascular and renal complications[5,58]. In cirrhotic patients with diabetes, the most recurrent cause of death is liver failure[4,19,58].

DM increases the severity and mortality of HCC

At present, type 2 DM is considered a risk factor for the occurrence of HCC. Hepatogenous diabetes together with hepatitis B and C virus infection and alcoholic liver cirrhosis increases the risk of HCC by 10-fold[1,2].

Patients with HCC and DM have a mortality risk higher than patients with HCC without DM. In another study involving 160 patients suffering from HCC, those who had DM had a 1-year mortality rate higher than those patients without DM. Additionally, they had more extensive disease[65].

Pathophysiologic mechanisms

The mechanisms by which diabetes worsens the clinical course of liver cirrhosis have not been clearly established. Firstly, DM accelerates liver fibrosis and inflammation

giving rise to more severe liver failure. Secondly, DM may potentiate the incidence of bacterial infections in cirrhotic patients which are associated with increased mortality[66,67].

In relation to the first mechanism, insulin resistance increases adipokine production (cytokines secreted by adipose tissue), such as leptin and TNF-α, which activates the inflammatory pathways that exacerbate liver damage[68]. In contrast, another cytokine produced by adipose tissue, adiponectin, is a regulator of insulin sensitivity and tissue inflammation[69]. A reduction in the adiponectin levels reflects peripheral and hepatic insulin resistance[70]. There has been speculation that hypoadiponectinemia may play a role in liver disease progression[70,71].

Regarding the second mechanism, DM may worsen immunodepression in cirrhotic patients thus increasing the incidence of severe infections which may have deleterious effect on liver function. It should be noted that cirrhotic patients with spontaneous bacterial peritonitis have a high hospital mortality rate due to sepsis, liver failure and hepatorenal syndrome. On the other hand, patients with esophageal variceal bleeding have a high incidence of infections that increase their in-hospital mortality rate[72]. Notwithstanding, it has not been established if DM increases the mortality rate in patients with other complications of cirrhosis.

In future, the precise mechanisms by which DM may worsen liver function should be clarified, since manipulation of these may be useful for reduction of complications.

CLINICAL IMPLICATIONS OF DM IN THE COURSE OF LIVER CIRRHOSIS AND TREATMENT OF DIABETES

Clinical manifestations

Clinical manifestations of DM in the early stages of cirrhosis are virtually absent. In a recently published study involving compensated cirrhotic patients with normal fasting serum glucose and without a family history of type 2 DM, up to 77% had DM or glucose intolerance diagnosed by means of OGTT. In 38% of cases, DM was subclinical[19]. As liver function deteriorates, the incidence of diabetes increases so that clinical diabetes may be seen as a marker of liver failure.

Hepatogenous diabetes has particular clinical characteristics: (1) unlike the hereditary type 2 DM, it is less frequently associated with risk factors such as age, body mass index, and family history of diabetes; (2) it is less frequently associated with retinopathy and cardiovascular and renal complications; (3) it is more frequently associated with hypoglycemic episodes as a result of impaired liver function[2,19].

Although the incidence of obesity, DM and metabolic syndrome have increased in the world, reaching epidemic proportions, the role of DM as a prognostic factor of morbidity and mortality in cirrhotic patients has been scarcely studied. In addition, the impact of early diagnosis and treatment of DM on the clinical course of cirrhosis is unknown.

Treatment

Few studies have evaluated what is the most efficacious therapy for DM in cirrhotic patients and what is the impact of treatment of DM on the clinical course of liver disease.

The treatment of DM of cirrhotic patients has particular characteristics that make it different from type 2 DM without liver disease: (1) about half the patients have malnutrition; (2) when clinical DM is diagnosed, the patient has advanced liver disease; (3) most of the oral hypoglycemic agents are metabolized in the liver; (4) patients often have episodes of hypoglycemia[3].

The initial treatment of patients suffering from mild to moderate hyperglycemia and compensated liver disease may be a lifestyle change, since at this stage the insulin resistance is a dominant factor. However, these therapeutic measures may be compromised by very restrictive diets, since they might aggravate malnutrition in some patients. On the other hand, physical exercise which improves insulin resistance may not be an appropriate measure in patients with active liver disease[15].

When DM manifests in advanced stages of liver disease, the use of oral hypoglycemic drugs may be required. However, most of these drugs are metabolized in the liver, therefore, the blood glucose levels during treatment shall be closely monitored in order to avoid hypoglycemia[73]. In these cases, biguanides, which reduce resistance to insulin, may be useful. Metformin is a biguanide that is relatively contraindicated in patients with advanced liver failure and patients who continue to ingest alcohol, because of the risk of lactic acidosis[74].

On the other hand, insulin secretagogues, despite the fact that they are safe drugs in patients with liver disease, probably are not useful, since they do not modify insulin resistance and patients with alcoholic cirrhosis often have pancreatic islet β-cell damage[75]. These patients have chronic compensatory hyperinsulinemia until the islet β-cells are exhausted.

Alpha-glucosidase inhibitors can be useful in patients suffering from liver cirrhosis, since their mechanism of action is to reduce carbohydrate absorption in the bowel, thus reducing the risk of postprandial hyperglycemia that is common in these patients. In a randomized, double-blind study involving 100 patients with compensated liver cirrhosis and insulin-treated DM, the control of postprandial and fasting blood glucose levels improved significantly with the use of acarbose, an alpha-glucosidase[76]. In another crossover placebo-controlled study involving patients with hepatic encephalopathy, acarbose produced a significant improvement in postprandial blood glucose level. Additionally, the patients had a reduction in plasma ammonia levels and an increase in the frequency of bowel movements[77]. The reduction in ammonia levels was probably the result of a decrease in the proliferation of intestinal proteolytic bacteria caused by bowel movement[77].

Thiazolidines may be particularly useful in cirrhotic patients with DM, since they increase the insulin sensitivity. However, troglitazone has been withdrawn from the market because of its potential hepatotoxic effects.

Nevertheless, rosiglitazone and pioglitazone appear to be safer drugs[78], but it is recommended that these drugs should not be initiated if there is evidence of active liver disease or if alanine transaminase levels are above 2.5 times the upper limit of normal. The use of these drugs should be monitored closely whenever necessary.

Insulin requirements in the cirrhotic patient with diabetes may vary. In patients with compensated cirrhosis, requirements may be greater compared to patients with decompensated cirrhosis, since insulin resistance predominates in the former while in the latter liver metabolism of insulin is greatly reduced. Therefore, therapy with insulin must be preferably performed in hospitalized patients with close monitoring of blood glucose levels for development of hypoglycemia[79].

Finally, liver transplantation rapidly normalizes glucose tolerance and insulin sensitivity. It is thought that this effect is due to an improvement in the hepatic clearance and peripheral glucose disposal. The latter effect could be secondary to a correction of chronic hyperinsulinemia[16,80]. It has been observed that liver transplantation, in reducing insulin resistance, cures hepatogenous diabetes in 67% of cirrhotic-diabetic patients. In 33% of patients diabetes was not corrected because of persistence of a reduced β-cell function measured by means of an OGTT. This abnormality would make these patients eventually eligible for combined islet transplantation[81].

FUTURE PERSPECTIVES IN RESEARCH OF DM IN LIVER CIRRHOSIS

Future research in the field of DM in cirrhotic patients should clarify the following issues: (1) the role of isolated type 2 DM in the genesis of chronic liver disease and the factors involved in this complication; (2) the impact of hepatogenous diabetes in the natural history of cirrhotic patients; (3) the impact of early diagnosis and treatment of hepatogenous diabetes (through OGTT) in reducing mortality; (4) the benefits of controlling the DM in the management of complications of liver cirrhosis; (5) the mechanisms by which hepatogenous diabetes increases morbidity and mortality of cirrhotic patients, as well as the impact of the manipulation of these mechanisms on the patients; (6) the establishment of clearer guidelines for management of diabetes in the cirrhotic patient.

Perhaps the combination of DM with the currently used scores (Child-Pugh and MELD scores) may enhance the sensitivity and the specificity for prediction of morbidity and mortality rates in cirrhotic patients.

REFERENCES

1 **Hickman IJ**, Macdonald GA. Impact of diabetes on the severity of liver disease. *Am J Med* 2007; **120**: 829-834
2 **El-Serag HB**, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* 2004; **126**: 460-468
3 **Tolman KG**, Fonseca V, Dalpiaz A, Tan MH. Spectrum of liver disease in type 2 diabetes and management of patients with diabetes and liver disease. *Diabetes Care* 2007; **30**: 734-743
4 **El-Serag HB**, Everhart JE. Diabetes increases the risk of acute hepatic failure. *Gastroenterology* 2002; **122**: 1822-1828
5 **Holstein A**, Hinze S, Thiessen E, Plaschke A, Egberts EH. Clinical implications of hepatogenous diabetes in liver cirrhosis. *J Gastroenterol Hepatol* 2002; **17**: 677-681
6 **Picardi A**, D'Avola D, Gentilucci UV, Galati G, Fiori E, Spataro S, Afeltra A. Diabetes in chronic liver disease: from old concepts to new evidence. *Diabetes Metab Res Rev* 2006; **22**: 274-283
7 **Custro N**, Carroccio A, Ganci A, Scafidi V, Campagna P, Di Prima L, Montalto G. Glycemic homeostasis in chronic viral hepatitis and liver cirrhosis. *Diabetes Metab* 2001; **27**: 476-481
8 **Postic C**, Dentin R, Girard J. Role of the liver in the control of carbohydrate and lipid homeostasis. *Diabetes Metab* 2004; **30**: 398-408
9 **Barthel A**, Schmoll D. Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol Endocrinol Metab* 2003; **285**: E685-E692
10 **Cotrozzi G**, Casini Raggi V, Relli P, Buzzelli G. [Role of the liver in the regulation of glucose metabolism in diabetes and chronic liver disease] *Ann Ital Med Int* 1997; **12**: 84-91
11 **Tappy L**, Minehira K. New data and new concepts on the role of the liver in glucose homeostasis. *Curr Opin Clin Nutr Metab Care* 2001; **4**: 273-277
12 **Nielsen MF**, Caumo A, Aagaard NK, Chandramouli V, Schumann WC, Landau BR, Schmitz O, Vilstrup H. Contribution of defects in glucose uptake to carbohydrate intolerance in liver cirrhosis: assessment during physiological glucose and insulin concentrations. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G1135-G1143
13 **Steppan CM**, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. *Nature* 2001; **409**: 307-312
14 **Petrides AS**, Vogt C, Schulze-Berge D, Matthews D, Strohmeyer G. Pathogenesis of glucose intolerance and diabetes mellitus in cirrhosis. *Hepatology* 1994; **19**: 616-627
15 **Petrides AS**, Stanley T, Matthews DE, Vogt C, Bush AJ, Lambeth H. Insulin resistance in cirrhosis: prolonged reduction of hyperinsulinemia normalizes insulin sensitivity. *Hepatology* 1998; **28**: 141-149
16 **Merli M**, Leonetti F, Riggio O, Valeriano V, Ribaudo MC, Strati F, Tisone G, Casciani CU, Capocaccia L. Glucose intolerance and insulin resistance in cirrhosis are normalized after liver transplantation. *Hepatology* 1999; **30**: 649-654
17 **Petrides AS**, Groop LC, Riely CA, DeFronzo RA. Effect of physiologic hyperinsulinemia on glucose and lipid metabolism in cirrhosis. *J Clin Invest* 1991; **88**: 561-570
18 **Lecube A**, Hernandez C, Genesca J, Esteban JI, Jardi R, Simo R. High prevalence of glucose abnormalities in patients with hepatitis C virus infection: a multivariate analysis considering the liver injury. *Diabetes Care* 2004; **27**: 1171-1175
19 **Nishida T**, Tsuji S, Tsujii M, Arimitsu S, Haruna Y, Imano E, Suzuki M, Kanda T, Kawano S, Hiramatsu N, Hayashi N, Hori M. Oral glucose tolerance test predicts prognosis of patients with liver cirrhosis. *Am J Gastroenterol* 2006; **101**: 70-75
20 **Di Bisceglie AM**. What every hepatologist should know about endocrinology: obesity, diabetes, and liver disease. *Gastroenterology* 2004; **126**: 604-606
21 **Wideroff L**, Gridley G, Mellemkjaer L, Chow WH, Linet M, Keehn S, Borch-Johnsen K, Olsen JH. Cancer incidence in a population-based cohort of patients hospitalized with diabetes mellitus in Denmark. *J Natl Cancer Inst* 1997; **89**: 1360-1365
22 **Fujino Y**, Mizoue T, Tokui N, Yoshimura T. Prospective study of diabetes mellitus and liver cancer in Japan. *Diabetes Metab Res Rev* 2001; **17**: 374-379
23 **Tazawa J**, Maeda M, Nakagawa M, Ohbayashi H, Kusano F, Yamane M, Sakai Y, Suzuki K. Diabetes mellitus may be associated with hepatocarcinogenesis in patients with chronic hepatitis C. *Dig Dis Sci* 2002; **47**: 710-715
24 **Donadon V**, Balbi M, Casarin P, Vario A, Alberti A.

Association between hepatocellular carcinoma and type 2 diabetes mellitus in Italy: Potential role of insulin. *World J Gastroenterol* 2008; **14**: 5695-5700
25 **Veldt BJ**, Chen W, Heathcote EJ, Wedemeyer H, Reichen J, Hofmann WP, de Knegt RJ, Zeuzem S, Manns MP, Hansen BE, Schalm SW, Janssen HL. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. *Hepatology* 2008; **47**: 1856-1862
26 **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387-1395
27 **Angulo P**. GI epidemiology: nonalcoholic fatty liver disease. *Aliment Pharmacol Ther* 2007; **25**: 883-889
28 **Caldwell SH**, Oelsner DH, Iezzoni JC, Hespenheide EE, Battle EH, Driscoll CJ. Cryptogenic cirrhosis: clinical characterization and risk factors for underlying disease. *Hepatology* 1999; **29**: 664-669
29 **Tellez-Avila FI**, Sanchez-Avila F, Garcia-Saenz-de-Sicilia M, Chavez-Tapia NC, Franco-Guzman AM, Lopez-Arce G, Cerda-Contreras E, Uribe M. Prevalence of metabolic syndrome, obesity and diabetes type 2 in cryptogenic cirrhosis. *World J Gastroenterol* 2008; **14**: 4771-4775
30 **Chalasani N**, Gorski JC, Asghar MS, Asghar A, Foresman B, Hall SD, Crabb DW. Hepatic cytochrome P450 2E1 activity in nondiabetic patients with nonalcoholic steatohepatitis. *Hepatology* 2003; **37**: 544-550
31 **Pessayre D**, Fromenty B, Mansouri A. Mitochondrial injury in steatohepatitis. *Eur J Gastroenterol Hepatol* 2004; **16**: 1095-1105
32 **Crespo J**, Cayon A, Fernandez-Gil P, Hernandez-Guerra M, Mayorga M, Dominguez-Diez A, Fernandez-Escalante JC, Pons-Romero F. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology* 2001; **34**: 1158-1163
33 **Sanyal AJ**. AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology* 2002; **123**: 1705-1725
34 **Bertolani C**, Marra F. The role of adipokines in liver fibrosis. *Pathophysiology* 2008; **15**: 91-101
35 **Buzzelli G**, Chiarantini E, Cotrozzi G, Relli P, Matassi L, Romanelli RG, Gentilini P. Estimate of prevalence of glucose intolerance in chronic liver disease. Degree of agreement among some diagnostic criteria. *Liver* 1988; **8**: 354-359
36 **Niederau C**, Fischer R, Purschel A, Stremmel W, Haussinger D, Strohmeyer G. Long-term survival in patients with hereditary hemochromatosis. *Gastroenterology* 1996; **110**: 1107-1119
37 **Del Vecchio Blanco C**, Gentile S, Marmo R, Carbone L, Coltorti M. Alterations of glucose metabolism in chronic liver disease. *Diabetes Res Clin Pract* 1990; **8**: 29-36
38 **Harrison SA**. Liver disease in patients with diabetes mellitus. *J Clin Gastroenterol* 2006; **40**: 68-76
39 **Angulo P**, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology* 1999; **30**: 1356-1362
40 **Romero-Gomez M**. Insulin resistance and hepatitis C. *World J Gastroenterol* 2006; **12**: 7075-7080
41 **Qureshi K**, Abrams GA. Metabolic liver disease of obesity and role of adipose tissue in the pathogenesis of nonalcoholic fatty liver disease. *World J Gastroenterol* 2007; **13**: 3540-3553
42 **Hatzitolios A**, Savopoulos C, Lazaraki G, Sidiropoulos I, Haritanti P, Lefkopoulos A, Karagiannopoulou G, Tzioufa V, Dimitrios K. Efficacy of omega-3 fatty acids, atorvastatin and orlistat in non-alcoholic fatty liver disease with dyslipidemia. *Indian J Gastroenterol* 2004; **23**: 131-134
43 **Mehta SH**, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL. Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. *Ann Intern Med* 2000; **133**: 592-599
44 **Knobler H**, Schihmanter R, Zifroni A, Fenakel G, Schattner A. Increased risk of type 2 diabetes in noncirrhotic patients with chronic hepatitis C virus infection. *Mayo Clin Proc* 2000; **75**: 355-359
45 **Anty R**, Gelsi E, Giudicelli J, Marine-Barjoan E, Gual P, Benzaken S, Saint-Paul MC, Sadoul JL, Huet PM, Tran A. Glucose intolerance and hypoadiponectinemia are already present in lean patients with chronic hepatitis C infected with genotype non-3 viruses. *Eur J Gastroenterol Hepatol* 2007; **19**: 671-677
46 **Lecube A**, Hernandez C, Genesca J, Simo R. Proinflammatory cytokines, insulin resistance, and insulin secretion in chronic hepatitis C patients: A case-control study. *Diabetes Care* 2006; **29**: 1096-1101
47 **Moucari R**, Asselah T, Cazals-Hatem D, Voitot H, Boyer N, Ripault MP, Sobesky R, Martinot-Peignoux M, Maylin S, Nicolas-Chanoine MH, Paradis V, Vidaud M, Valla D, Bedossa P, Marcellin P. Insulin resistance in chronic hepatitis C: association with genotypes 1 and 4, serum HCV RNA level, and liver fibrosis. *Gastroenterology* 2008; **134**: 416-423
48 **Hui JM**, Sud A, Farrell GC, Bandara P, Byth K, Kench JG, McCaughan GW, George J. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression [corrected]. *Gastroenterology* 2003; **125**: 1695-1704
49 **Romero-Gomez M**, Del Mar Viloria M, Andrade RJ, Salmeron J, Diago M, Fernandez-Rodriguez CM, Corpas R, Cruz M, Grande L, Vazquez L, Munoz-De-Rueda P, Lopez-Serrano P, Gila A, Gutierrez ML, Perez C, Ruiz-Extremera A, Suarez E, Castillo J. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology* 2005; **128**: 636-641
50 **Sanyal AJ**, Chand N, Comar K, Mirshahi F. Hyperinsulinemia blocks the inhibition of hepatitis C virus (HCV) replication by interferon: a potential mechanism for failure of interferon therapy in subjects with HCV and nonalcoholic liver disease. *Hepatology* 2004; **40** (4 Suppl 1): 179A
51 **Giordanino C**, Bugianesi E, Smedile A, Ciancio A, Abate ML, Olivero A, Pellicano R, Cassader M, Gambino R, Bo S, Ciccone G, Rizzetto M, Saracco G. Incidence of type 2 diabetes mellitus and glucose abnormalities in patients with chronic hepatitis C infection by response to treatment: results of a cohort study. *Am J Gastroenterol* 2008; **103**: 2481-2487
52 **Zein NN**, Abdulkarim AS, Wiesner RH, Egan KS, Persing DH. Prevalence of diabetes mellitus in patients with end-stage liver cirrhosis due to hepatitis C, alcohol, or cholestatic disease. *J Hepatol* 2000; **32**: 209-217
53 **Wei M**, Gibbons LW, Mitchell TL, Kampert JB, Blair SN. Alcohol intake and incidence of type 2 diabetes in men. *Diabetes Care* 2000; **23**: 18-22
54 **Adams PC**, Kertesz AE, Valberg LS. Clinical presentation of hemochromatosis: a changing scene. *Am J Med* 1991; **90**: 445-449
55 **Greco AV**, Mingrone G, Mari A, Capristo E, Manco M, Gasbarrini G. Mechanisms of hyperinsulinaemia in Child's disease grade B liver cirrhosis investigated in free living conditions. *Gut* 2002; **51**: 870-875
56 **Kim MG**, Choi WC. [Differential diagnosis of diabetes mellitus caused by liver cirrhosis and other type 2 diabetes mellitus] *Korean J Hepatol* 2006; **12**: 524-529
57 **de Marco R**, Locatelli F, Zoppini G, Verlato G, Bonora E, Muggeo M. Cause-specific mortality in type 2 diabetes. The Verona Diabetes Study. *Diabetes Care* 1999; **22**: 756-761
58 **Bianchi G**, Marchesini G, Zoli M, Bugianesi E, Fabbri A, Pisi E. Prognostic significance of diabetes in patients with cirrhosis. *Hepatology* 1994; **20**: 119-125
59 **Trombetta M**, Spiazzi G, Zoppini G, Muggeo M. Review article: type 2 diabetes and chronic liver disease in the Verona diabetes study. *Aliment Pharmacol Ther* 2005; **22** Suppl 2: 24-27
60 **Hourigan LF**, Macdonald GA, Purdie D, Whitehall VH, Shorthouse C, Clouston A, Powell EE. Fibrosis in chronic hepatitis C correlates significantly with body mass index and steatosis. *Hepatology* 1999; **29**: 1215-1219

61 **Taura N**, Ichikawa T, Hamasaki K, Nakao K, Nishimura D, Goto T, Fukuta M, Kawashimo H, Fujimoto M, Kusumoto K, Motoyoshi Y, Shibata H, Abiru N, Yamasaki H, Eguchi K. Association between liver fibrosis and insulin sensitivity in chronic hepatitis C patients. *Am J Gastroenterol* 2006; **101**: 2752-2759

62 **Flores-Rendon AR**, Gonzalez-Gonzalez JA, Garcia-Compean D, Maldonado-Garza HJ, Garza-Galindo AA. Model for end stage of liver disease (MELD) is better than the Child-Pugh score for predicting in-hospital mortality related to esophageal variceal bleeding. *Ann Hepatol* 2008; **7**: 230-234

63 **Durand F**, Valla D. Assessment of the prognosis of cirrhosis: Child-Pugh versus MELD. *J Hepatol* 2005; **42** Suppl: S100-S107

64 **Moreau R**, Delegue P, Pessione F, Hillaire S, Durand F, Lebrec D, Valla DC. Clinical characteristics and outcome of patients with cirrhosis and refractory ascites. *Liver Int* 2004; **24**: 457-464

65 **Amarapurkar DN**, Patel ND, Kamani PM. Impact of diabetes mellitus on outcome of HCC. *Ann Hepatol* 2008; **7**: 148-151

66 **Garcia-Tsao G**. Bacterial infections in cirrhosis: treatment and prophylaxis. *J Hepatol* 2005; **42** Suppl: S85-S92

67 **Cheruvattath R**, Balan V. Infections in Patients With End-stage Liver Disease. *J Clin Gastroenterol* 2007; **41**: 403-411

68 **Roden M**. Mechanisms of Disease: hepatic steatosis in type 2 diabetes--pathogenesis and clinical relevance. *Nat Clin Pract Endocrinol Metab* 2006; **2**: 335-348

69 **Whitehead JP**, Richards AA, Hickman IJ, Macdonald GA, Prins JB. Adiponectin--a key adipokine in the metabolic syndrome. *Diabetes Obes Metab* 2006; **8**: 264-280

70 **Jonsson JR**, Moschen AR, Hickman IJ, Richardson MM, Kaser S, Clouston AD, Powell EE, Tilg H. Adiponectin and its receptors in patients with chronic hepatitis C. *J Hepatol* 2005; **43**: 929-936

71 **Svegliati-Baroni G**, Ridolfi F, Di Sario A, Casini A, Marucci L, Gaggiotti G, Orlandoni P, Macarri G, Perego L, Benedetti A, Folli F. Insulin and insulin-like growth factor-1 stimulate proliferation and type I collagen accumulation by human hepatic stellate cells: differential effects on signal transduction pathways. *Hepatology* 1999; **29**: 1743-1751

72 **Hou MC**, Lin HC, Liu TT, Kuo BI, Lee FY, Chang FY, Lee SD. Antibiotic prophylaxis after endoscopic therapy prevents rebleeding in acute variceal hemorrhage: a randomized trial. *Hepatology* 2004; **39**: 746-753

73 **Marks V**, Teale JD. Drug-induced hypoglycemia. *Endocrinol Metab Clin North Am* 1999; **28**: 555-577

74 **Nair S**, Diehl AM, Wiseman M, Farr GH Jr, Perrillo RP. Metformin in the treatment of non-alcoholic steatohepatitis: a pilot open label trial. *Aliment Pharmacol Ther* 2004; **20**: 23-28

75 **Choudhury S**, Hirschberg Y, Filipek R, Lasseter K, McLeod JF. Single-dose pharmacokinetics of nateglinide in subjects with hepatic cirrhosis. *J Clin Pharmacol* 2000; **40**: 634-640

76 **Gentile S**, Turco S, Guarino G, Oliviero B, Annunziata S, Cozzolino D, Sasso FC, Turco A, Salvatore T, Torella R. Effect of treatment with acarbose and insulin in patients with non-insulin-dependent diabetes mellitus associated with non-alcoholic liver cirrhosis. *Diabetes Obes Metab* 2001; **3**: 33-40

77 **Gentile S**, Guarino G, Romano M, Alagia IA, Fierro M, Annunziata S, Magliano PL, Gravina AG, Torella R. A randomized controlled trial of acarbose in hepatic encephalopathy. *Clin Gastroenterol Hepatol* 2005; **3**: 184-191

78 **Lebovitz HE**, Kreider M, Freed MI. Evaluation of liver function in type 2 diabetic patients during clinical trials: evidence that rosiglitazone does not cause hepatic dysfunction. *Diabetes Care* 2002; **25**: 815-821

79 **Petrides AS**. Hepatogenic diabetes: pathophysiology, therapeutic options and prognosis. *Z Gastroenterology* 1999; **16** (Suppl 1): 15-21

80 **Blanco JJ**, Herrero JI, Quiroga J, Sangro B, Gomez-Manero N, Pardo F, Cienfuegos JA, Prieto J. Liver transplantation in cirrhotic patients with diabetes mellitus: midterm results, survival, and adverse events. *Liver Transpl* 2001; **7**: 226-233

81 **Perseghin G**, Mazzaferro V, Sereni LP, Regalia E, Benedini S, Bazzigaluppi E, Pulvirenti A, Leao AA, Calori G, Romito R, Baratti D, Luzi L. Contribution of reduced insulin sensitivity and secretion to the pathogenesis of hepatogenous diabetes: effect of liver transplantation. *Hepatology* 2000; **31**: 694-703

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TOPIC HIGHLIGHT

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Contrast-enhanced 3D ultrasound in the radiofrequency ablation of liver tumors

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Abstract

Liver metastases and hepatocellular carcinomas are two of the most common causes of cancer deaths in the world. Radiofrequency ablation (RFA) is a well recognized, effective and minimally invasive means of treating malignant hepatic tumors. This article describes the use of contrast-enhanced 3D ultrasound (CE-3DUS) in the staging, targeting and follow-up of patients with liver tumors undergoing RFA. In particular, its value in the management of large hepatic lesions will be illustrated. Current limitations of CE-3DUS and future developments in the technique will also be discussed. In summary, CE-3DUS is useful in the RFA of liver tumors with improved detection and display of occult lesions and recurrence, in the assessment of lesional geometry and orientation for a more accurate planning and guidance of multiple RFA needle electrodes in large tumors and in the evaluation of residual or recurrent disease within the immediate and/or subsequent follow-up periods.

Key words: Liver tumors; Radiofrequency ablation; Contrast enhanced 3D ultrasound

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INTRODUCTION

Colorectal cancer liver metastases and hepatocellular carcinomas are the two most common malignancies of the liver, associated with a dismal outcome of zero survival at 5-year if left untreated. Radiofrequency ablation (RFA) is rapidly emerging as one of the most popular, effective, minimally invasive, alternative therapeutic tool to hepatic surgery in the treatment of these liver malignancies. Conventional 2-dimensional (2D) unenhanced ultrasound (US) guidance of RFA is limited due to its lower sensitivity when compared with the referal modalities, such as contrast enhanced computerized tomography (CT) or magnetic resonance imaging (MRI). Recent advances in non-linear imaging modes and developments in 3-dimensional (3D) mechanical and electronic ultrasound probes have led to a marked improvement in the real-time contrast enhanced volumetric imaging with potential impact in the detection, planning and targeting strategy of RFA needle electrodes in the treatment beyond small lesions. The objective of this article is to demonstrate the usefulness of contrast-enhanced 3D ultrasound (CE-3DUS) in the radiofrequency ablation of these liver tumors. The indications for RFA of metastases and hepatocellular carcinomas will be reviewed within the appropriate clinical settings. The applications of CE-3DUS in all the aspects of staging, planning, targeting and follow-up of RFA are described and illustrated.

CLINICAL BACKGROUND

In the Western World, colorectal cancer accounts for

14 and 16 percent of cancer deaths in men and women, respectively, with approximately 25% of patients having liver involvement at the time of initial presentation and up to 50% will develop hepatic metastases during the course of their disease[1,2]. For patients with colorectal liver metastases, surgical resection is the treatment of choice, but only 10%-20% of patients are initially candidates for potentially curative resection; resection should be considered if there is no unresectable extra-hepatic disease, all liver deposits can be resected with a free clearance margin of 1 cm, and there is adequate liver reserve. The five-year survival rates vary from 25%-40%[3,4]. Seventy five percent of those who undergo liver resection will develop recurrence and of these, the liver is involved in 50%. Sixty five to eighty five percent of all recurrences appear within the first 2 years[5]. Repeat liver resection in these patients still has a 5-year survival of 30 to 40 percent. Whilst the post-operative mortality or morbidity following repeated hepatectomy is comparable to those of single hepatectomy, they are not entirely negligible and hepatectomy is associated with significant monetary expense[5,6].

The worldwide incidence of hepatocellular carcinoma is increasing and in particular within North America and Europe, which is progressively affecting younger patients. It is now believed that this is mainly attributed to the rise in hepatitis C viral infection, as the rates associated with alcoholic cirrhosis and hepatitis B virus infection have remained stable[7]. The disease is extremely lethal with median survival rates of untreated symptomatic cases ranging between 4 to 6 mo. Patients with even small tumors also carry a significant mortality as less than 50% will survive 5 years despite undergoing apparently curative resection. Patients with early stage hepatocellular carcinoma should be offered surgical therapeutic options of transplantation or resection[8]. Transplantation offers a 4-year overall survival rate of 75% and a 4-year recurrence free survival rate of 83%. However, few would benefit from transplantation given the shortage of living donors and the eligibility of patients to the "Milan criteria" for transplantation (i.e. decompensated cirrhosis, solitary tumor smaller than 5 cm and up to 3 lesions smaller than 3 cm)[9]. Similarly, less than 5% of cirrhotic patients with hepatocellular carcinoma would be suitable for hepatic resection under current criteria (those with limited tumour burden and relatively well preserved liver function).

Given the shortcomings of current surgical approach, an effective, minimally invasive and repeatable technique for the treatment of liver tumors could potentially impact favorably in the management of these patients.

RADIOFREQUENCY ABLATION

During the last decade, there has been considerable development of the ablative techniques for oncological applications including cryo-, radiofrequency-, microwave- or laser- ablation and high intensity focused ultrasound (HIFU). The development of radiofrequency ablation can be traced back to 1891 through the works of d'Arsonval[10]. In more recent years, with the additional refinements to the design and power of the equipments, it has emerged as the most popular tool for the destruction of hepatic as well as other malignant tissues. During the application of radiofrequency energy, a high frequency alternating current moves from the electrode in the immediate surrounding tissue and as the ions within the tissue attempt to follow the change in the direction of the alternating current, frictional heating of the tissue is generated. Tissue temperature can be elevated beyond 100 degrees centigrade, resulting in coagulation necrosis of the tissue. Precise control of the extent of tissue destruction can be achieved by adjusting local temperature and electrical resistance.

Radiofrequency ablation of liver tumors can be performed percutaneously, laparoscopically or as part of an open surgical procedure[11-13]. Ultrasound guidance remains the optimal method for accurate targeting of the tumors with the RFA needle electrode as it is mobile, more practical, readily available, rapid and cost-effective compared with CT or MR guidance. Despite the availability of screening facilities with the current advanced CT or MR modalities, RFA guidance with the latter modalities is limited for multiple lesional ablations during the same session requiring repeated large volumes of contrast administration to complete all imaging requirements; as such there is some hampering of the work flow for the whole ablative process. As a result many centers use a combination of both CT and US for the ablative procedure.

INDICATIONS FOR RADIOFREQUENCY ABLATION

Radiofrequency ablation is indicated in patients with disease limited to the liver who do not meet the criteria for surgical resectability for both hepatocellular carcinomas and liver metastases[14-15]. RFA is now offered to those who cannot undergo resection because of inadequate surgical margins, inadequate liver reserve, co-existing morbidity or patient choice and is performed with a curative intent as in surgical resection. RFA is also effective in the destruction of lesions localized adjacent to major vascular structures including the hepatic veins confluence with the inferior vena cava which would preclude resection. The blood flow in major vessels acts as a heat sink that protects the vascular endothelium from thermal injury whilst allowing complete coagulation of tissue immediately surrounding the blood vessel wall. However, RFA is avoided for peri-hilar lesions due to potential biliary damage leading to fistulous or stricturing complications. With the increasingly aggressive approach adopted by liver surgeons, open radiofrequency ablation is routinely combined with liver resection in the presence of multi-focal, bilateral metastases or upon the detection of unexpected additional lesion. In these cases hepatectomy is performed to deal with the main tumor bulk and any residual tumors that cannot be resected,

is treated with RFA. Nonetheless, standard surgical considerations still apply with no more than 70% of the liver volume is removed and particular attention has to be paid to patients with background liver cirrhosis with limited functional reserve.

The use of new effective systemic chemotherapy for the colorectal liver metastases has increased the potential of obtaining significant response to the point of enabling resectability. Studies have shown that of patients with unresectable disease who received second line neo-adjuvant therapy, up to 22% became resectable[16]. There is also a growing proportion of these patients who have had their disease down-staged, who are subsequently referred for radiofrequency ablation instead of surgical resection. The development of fairly extensive steatosis in 20% to 66% of these patients is among the main reason for the choice of RFA, as there is significant morbidity and mortality associated in these cases following liver resection[17,18]. Patients with large hepatocellular carcinomas are also now being considered for trans-arterial chemo-embolisation/trans-arterial embolization followed by RFA of any residual disease. However, there is as yet no evidence that these combination therapies with RFA leads to any survival benefit compared with standard clinical practice. The use of RFA in the treatment of other types of metastatic tumors to the liver have also been advocated; for example stable (6 mo) disease from breast, renal, melanoma or neuro-endocrine metastases[13,19].

IMAGING TASKS FOR RFA

Intra-operative ultrasonography (IOUS) has been shown to yield significant new information, not identified on pre-operative imaging, which determines resectability or changes the operative plan in up to 50% of patients; it is considered the gold standard thereby achieving universal usage[20-23]. However, traditionally CT and MRI have been used to routinely stage all patients with hepatocellular carcinomas and metastatic hepatic colorectal disease. Given the well-recognized limitations of conventional unenhanced ultrasound, its role in the liver staging process has been negligible. More recently, there has been increasing interest in the use of ultrasound contrast agents during the sonography of the liver to improve the detection of liver metastases. Ultrasound contrast agents consist of microbubbles of air or gases of low solubility, stabilized by a lipid, surfactant or polymer shell. Analogous to CT or MR, it is the relative distribution of the contrast agents between normal tissue and the lesion, which makes the lesion more visible and easier to characterized[24,25]. Recent advances in non-linear imaging in the form of pulse inversion together with power modulation modes, combined with the development of contrast agents with liver specificity, have markedly improved the sensitivity of sonography in the detection of small metastases, which may be equal to or even superior to that of CT or MR in some cases[26,27].

Whilst we are accustomed to viewing cross-sectional imaging in a two dimensional perspective, tumor staging, its treatment planning, targeting and assessment of therapeutic response would clearly benefit from a three dimensional morphological as well as functional imaging aspect. In that respect, as a result of technological innovations in non-linear modes (NLM) and new probe design, real time imaging with CE 3D-US is now a reality.

Liver staging

Almost all patients are referred for RFA, based on the CT and/or MR imaging findings; RFA would only be feasible under ultrasound guidance, if all the lesions identified on the referral imaging modalities could actually be detected on ultrasound itself. However, conventional ultrasound is well recognized to be limited in the detection of liver metastases and in the identification of hepatocellular carcinoma in a multi-nodular cirrhotic background. Furthermore, in patients with colorectal liver metastases who have had neo-adjuvant chemotherapy and subsequently referred for RFA, the difficulty of identifying all metastases is significantly increased due to widespread steatosis. Compared with conventional ultrasound, contrast-enhanced ultrasound (CE-US) has been shown to be highly accurate in determining the extent and distribution of tumor burden within the liver. Recent studies have shown that the sensitivity and accuracy of CE-US are comparable with those of CT and/or MR enhanced with liver specific contrast agents[26,27]. Within the RFA clinical setting, the sensitivity of ultrasound in the detection of metastases, HCCs and all lesions combined has been reported to be 42.9%, 66.7% and 51.4%, respectively; in comparison, the sensitivity for contrast enhanced ultrasound was 100% for all 3 groups[28]. Complete ultrasound guided RFA would have been impossible without the use of contrast agent in 60.4% as the lesions remained occult on the unenhanced ultrasound and of these, no lesions could be detected in 15.4%. To identify all lesions as seen on CT/MRI, contrast enhanced ultrasound was required in 88.9% patients with metastases compared with 41.3% of patients with hepatocellular carcinomas. Of the patients with metastases, 44.4% had been on second line systemic chemotherapy and all required contrast enhanced ultrasound to detect all lesions. No patient was subsequently referred for CT/MRI guided RFA. Follow-up CT/MRI confirmed successful targeting in all patients.

Moreover, in staging the liver for ablation, characterization of all the targeted lesions is important to ensure they are truly malignant; this is particularly so with referrals based on CT scans where hemangiomas may be misdiagnosed as metastases. In patients who have had systemic chemotherapy, there are potential problems with underestimation of the true extent of the disease as well as presence of pseudo-tumors due to diffuse areas of fatty infiltration and focal areas of fatty sparing, respectively. Contrast enhanced ultrasound is particularly useful in confirming the true nature of these benign lesions with real-time evaluation of the lesional micro-vascularization (Figures 1 and 2).

From a practical standpoint, scheduling for CT or

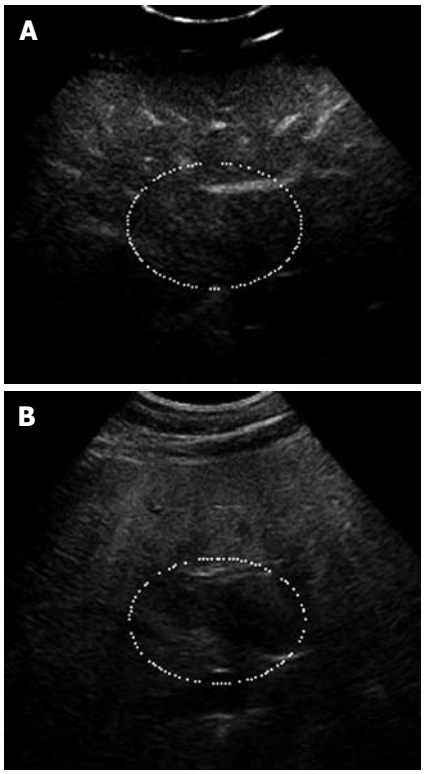

Figure 1 Focal fat sparing shown as iso-echogenicity as adjacent liver parenchyma. A: Late phase of CE-US; B: Hypo-echoic area on the fundamental mode.

MR scans within the 4-6 wk of the RFA procedure may not always be possible because of lack of availability and/or accessibility. With the benefit of its higher accuracy, contrast enhanced ultrasound also offers the flexibility in enabling re-staging immediately prior to the RFA procedure. Using standardized scanning protocols, CE 3D-US may even be superior to CE-US (2D) in the detection and display of these occult liver tumors as well as improving workflow. To ensure complete coverage of the liver, scanning protocols with CE-3DUS include wide angled automated sweeps at the epigastrium, sub-costally and the 3 intercostal spaces in the right upper quadrant of the abdomen, during the arterial, portal venous and late phases of the intravenous bolus injection of the contrast agent. The CE-3DUS set of data may be transferred onto a workstation or a PACS for archiving and reviewed subsequently; it can also be displayed in the same manner as CT or MRI, analyzed and then reported (Figure 3). The whole examination may be performed by the sonologist and the review can be carried out immediately online or subsequently by the sonologist or the Radiologist with improvement in the workflow. There are anecdotal reports of the superiority of CE-3DUS over baseline fundamental unenhanced 2D and 3D ultrasound in the detection and display of occult liver metastases (Figure 3). Furthermore, in dealing with larger tumors and in particular local recurrence, CE-3DUS can define more clearly the biologically active target tumor volume. This is supported by recent preliminary study using a 3D Shape based analysis of CT scans showing that the technique may be useful

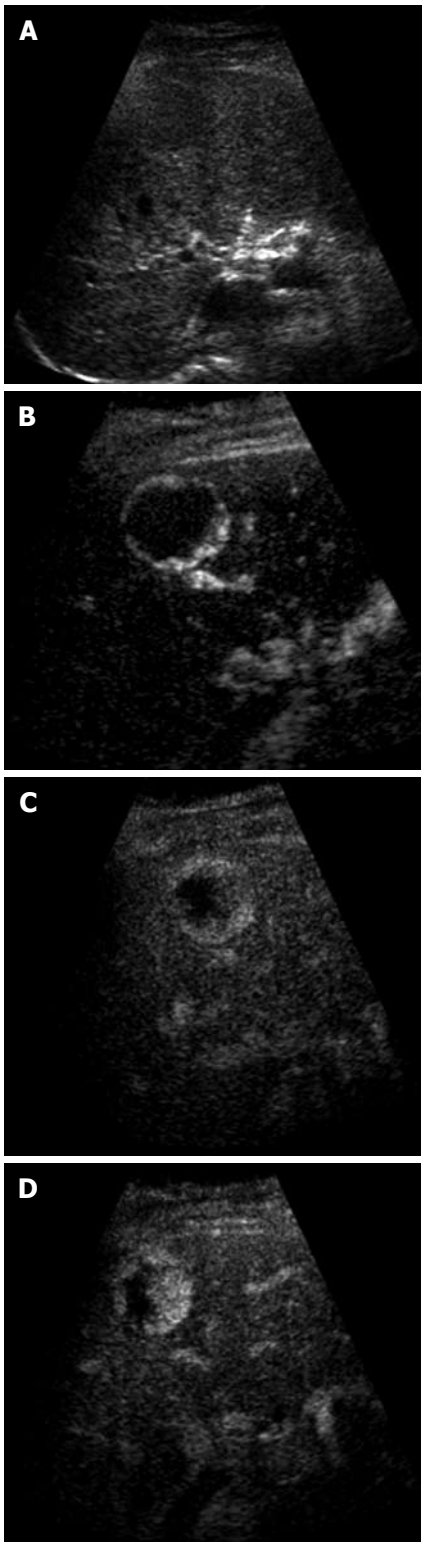

Figure 2 Haemangioma. A: Baseline focal hypo-echoic lesion; B: Arterial phase showing peripheral rim enhancement; C: Portal venous phase showing peripheral globular rim enhancement; D: Late phase showing progressive centripetal filling-in which is characteristic for hemangioma.

in assessing RFA ablation results in revealing earlier recurrences unsuspected clinically[29].

Assessment of tumour size and geometry

The selection of the RFA needle electrodes and the appropriate ablation protocols depend on the size of

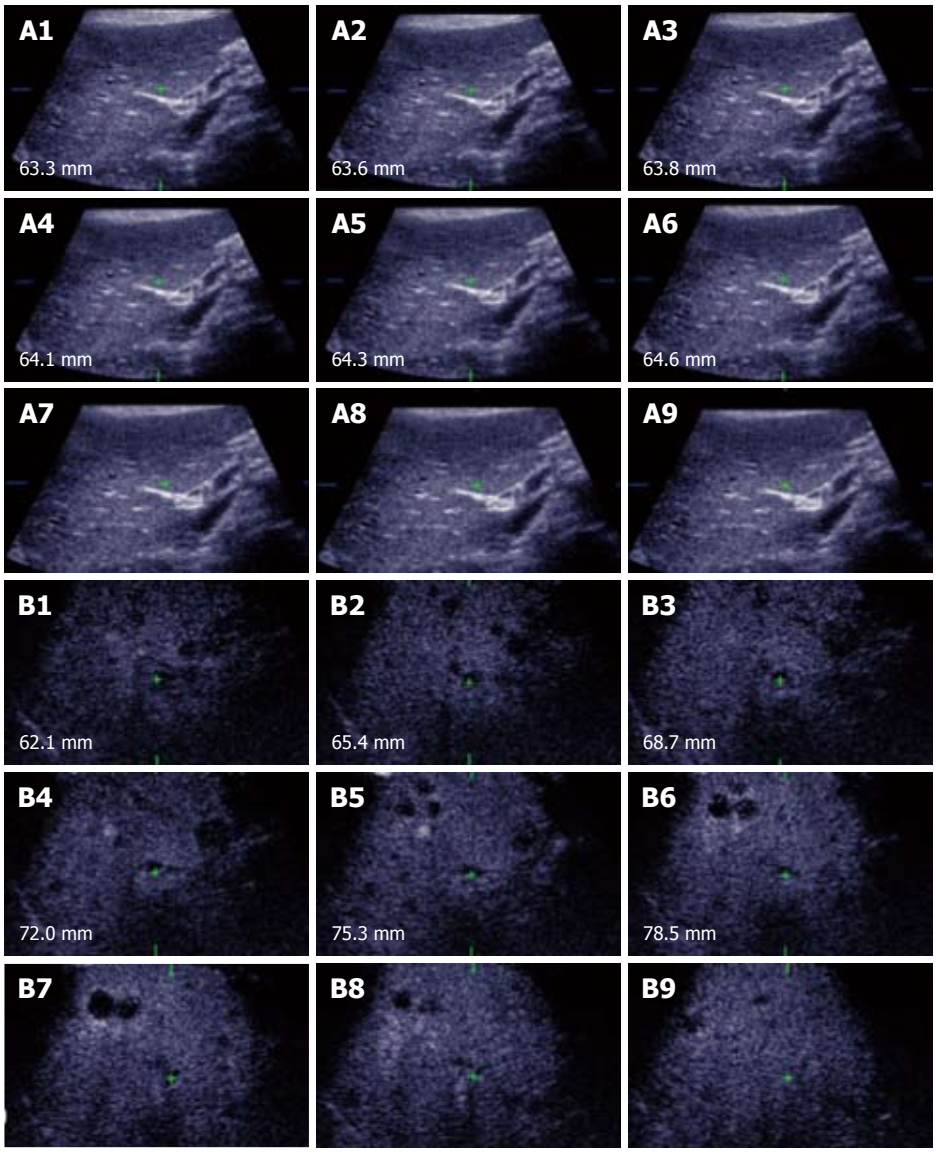

Figure 3 Unenhanced ultrasound and contrast enhanced 3D US. A: Unenhanced Fundamental mode ultrasound showing apparently normal liver; B: Contrast enhanced 3D US displayed as axial scans showing numerous occult metastases appearing as filling defects in the late phase.

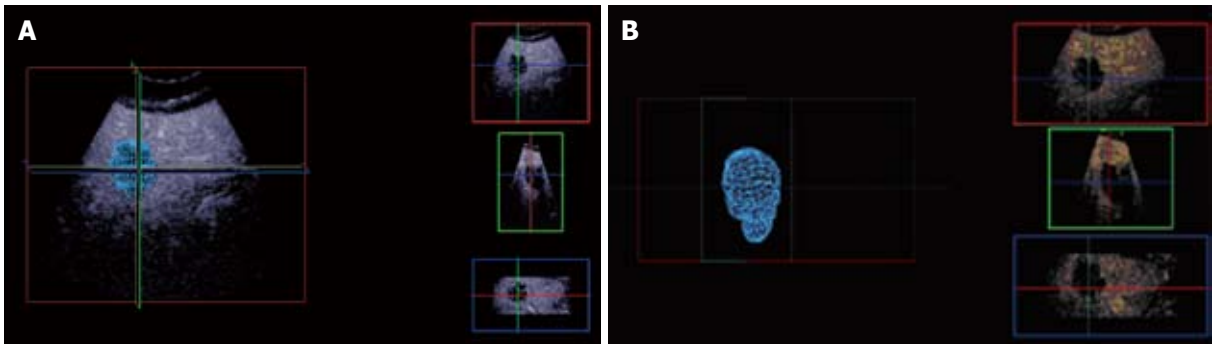

Figure 4 Multi-planar reconstruction. A: Tumor appears to be spherical in tumor modeling; B: Background liver subtraction shows geometry of the tumor model and long axis.

the tumor to be ablated. When the tumor is small (2 to 3 cm), its geometry is usually spherical. However, larger (> 3 cm) tumours may be elliptical. In locally advanced disease there may be aggregates of "daughter" hepatocellular carcinomas or "satellite" metastases merging as they grow resulting into lobular masses. Unenhanced and CE-3DUS assessment of the tumor geometry and determination of the lesional long axis is required to plan for the RFA targeting in order to restrict the number of RFA needle electrode insertions to the bare minimum to avoid seedling and potential complications (Figure 4). If the tumor is elongated, 3D assessment of the tumor geometry and its orientation relative to the probe position enables the planning of the insertion of the RFA needle electrode along the center of the tumor's long axis to treat the lower half of the

lesion first and subsequent withdrawal of the electrode to ablate the proximal residual tumor mass with a single puncture approach (Figure 4B).

Accurate delineation of the viable tumor margins in 3 orthogonal planes is a key in determining the true size of the lesion. Analogous to liver resection, ablation of the 0.5 to 1.0 cm layer of "normal" liver parenchyma around the tumor margin is important to limit subsequent local recurrence. Conventional US may be limited in delineating the true viable tumor margin; on the other hand CE-3DUS can accurately demonstrate the normal liver/tumor margin. Whilst this may not be critical for small lesions, with larger lesions a 5 mm error may significantly increase the risk of leaving residual disease. The importance of the true delineation and geometry is also largely related to the limited ablative capability of current RFA single or cluster needle electrodes which will produce at best a sphere of coagulation necrosis of 5 cm in diameter. Once the 3D data set has been acquired, the measurement of the tumor diameter in all 3 orthogonal planes determines the appropriate RFA needle electrodes.

Volume of the tumor can also be calculated automatically or manually depending on the software available. This can be done automatically following CE-3DUS using an "auto stacked contours" system, which automatically determines the tumor/normal liver border. First all 3 orthogonal planes are aligned into the center of the mass. The borders of the tumor are marked, and the number of slices for tracing the contours can be selected. Each contour is mapped and the tumor model created and its volume calculated automatically. Without the use of contrast, there is no automatic delineation of the tumor/normal liver border and the stacking of the tumor contours needs to be done manually which is more time consuming.

Targeting of tumors

Conventional ultrasound (2D) has been shown to be an excellent real-time tool in the placement of biopsy needles and RFA needle electrodes. Its limitation in the detection of occult liver tumors is well recognized compared with contrast enhanced CT and MR. Without the use of ultrasound contrast agents, these occult tumors could be localized using adjacent anatomical landmarks and then targeted blindly (i.e. without actual visualization of the tumor). CE-US facilitates the identification of these occult tumors for biopsy and/or for ablation. However, CE-US relies on the use of non-linear imaging mode to depict the contrast enhancement of the normal liver parenchyma with the malignant tumors appearing as filling defects in the late phase. Non-linear imaging mode is highly effective in subtracting native tissue linear echoes including those of the target biopsy needle or RFA needle electrodes. Whilst CE-US will identify the occult tumors, visualization of the biopsy needles and RFA needle electrodes is difficult on the non-linear imaging mode at low output power (Mechanical index). In the past, one had to switch between the non-linear imaging mode and fundamental modes screen to identify the position of the tumor and needle, respectively. However, with the advent of the side by side dual screen with the low MI fundamental mode and non-linear imaging mode simultaneously displaying the needle and the occult lesion, respectively, accurate real time targeting of occult lesions has been facilitated and significantly improves the work flow obviating the need to resort to CT or MRI scan guidance (Figure 5).

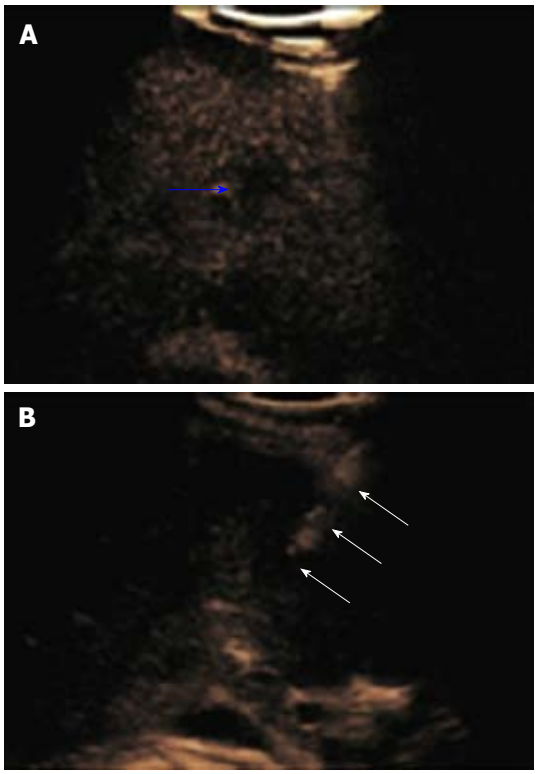

Figure 5 Side-by-side screen. Blue arrow points to small occult metastasis only seen on the CE-US non-linear imaging mode (A), whilst the open arrows point to the RFA needle electrode trajectory towards the occult metastasis, which is only visualized on the fundamental US scan (B).

With 2D US data acquisition, it is difficult to visualize mentally the 3D spatial aspects of the tumor and its specific relation to the surrounding hepatic vascular structures. CE-3DUS enables the modelling of the tumor geometry and determination of its long axis as well as its spatial relationship with the adjacent hepatic vascular anatomy and sometimes the extra-hepatic vital structures when the tumor is sub-capsular in location. The placement of the RFA needle electrodes can then be performed with reference to the probe position with the aid of a needle-guide or free-hand control (Figure 4). Furthermore, the added information provided by CE-3DUS also enables a more aggressive approach to the ablation of larger lesions beyond usage of a singular RFA needle electrode. Placement of multiple RFA needle electrodes will create coagulation necrosis even beyond the 7 cm (Figure 6). But the deployment of these multiple RFA needle electrodes needs to be accurate and is facilitated with CE-3DUS planning and guidance.

There are reports of improved needle localization

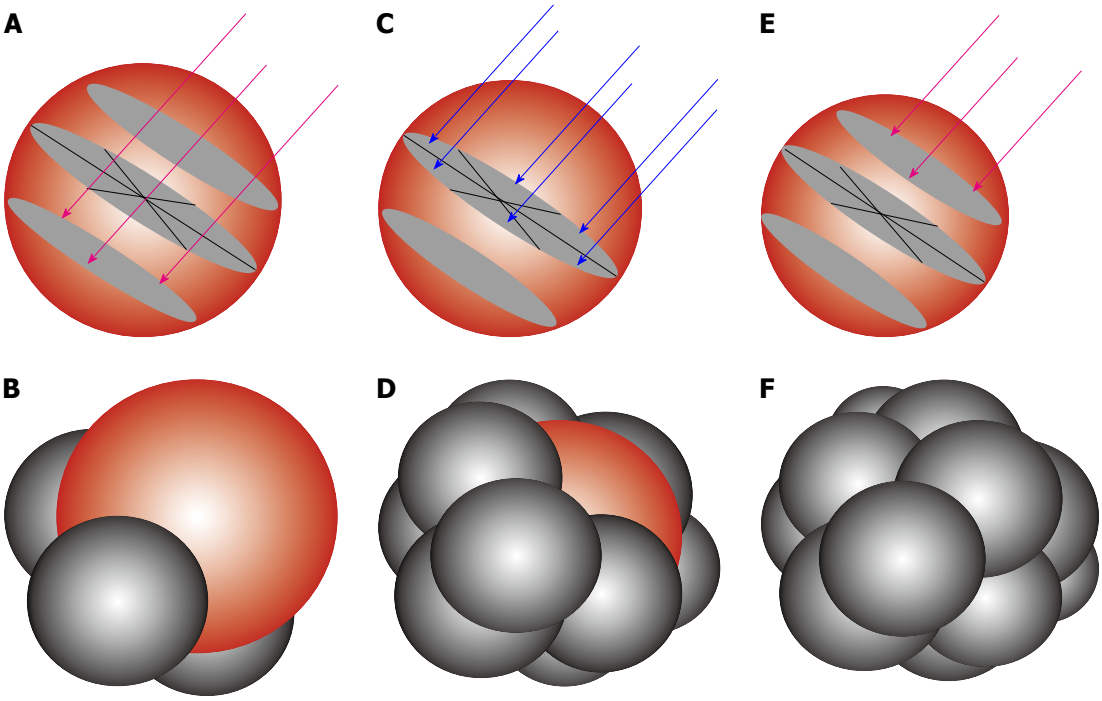

Figure 6 RFA needle electrode. Modeling to show the placement of the RFA needle electrodes to enable 12 overlapping spheres to give an equivalent of 7.5 cm diameter target sphere. (A, B) lower pole ablation with 3 RFA needle electrodes (red arrows) to create 3 overlapping ablation spheres (C, D) middle row ablation with 6 RFA needle electrodes (blue arrows) to create 6 overlapping ablation spheres and (E, F) upper pole ablation with retraction of the first 3 RFA electrodes to complete the target sphere of 7.5 cm.

Figure 7 RFA needle electrode with expandable antennas.

and guidance during biopsy using unenhanced 3D-US[30]. Earlier studies using unenhanced 3D-US to guide RFA needle electrodes have shown an increase in the confidence of the operator as well as a more accurate positioning of the electrodes in the majority of cases when compared with conventional 2D ultrasound guidance[31,32]. With regard to the accurate placement of RFA needle electrodes with expandable multiple antennas (Figure 7), particular attention needs to be observed (1) that the expandable antennas are deployed uniformly and symmetrically within the tumor to ensure margin (2) with lesions adjacent to vascular structures and sub-capsular location, that the latter are not punctured. 3D US is particularly valuable in the depiction of the safe deployment of these expandable antennas with the use of the elevation plane. 3D US enables the visualization of the RFA needle electrodes and the tumor simultaneously in the 3 orthogonal planes, among which the coronal and sagittal planes are aligned along the RFA needle electrode long axis whilst the elevation plane being perpendicular to it (Figures 8-10). Through translation or rotation within the acquired 3D volume, all regions of interest can be viewed from arbitrary orientations without any restriction of the transducer axis. The placement of multiple RFA needle electrodes can be planned following acquisition of the 3D volume data and accurate measurement of the distance between the electrodes and the tumor margin can be performed in advanced limiting the danger of unnecessary repeated electrode punctures through "trial and error" (Figure 11).

Monitoring of response

To assess the initial response to treatment, CE-3DUS can be performed only at 7 to 10 min after the end of RFA to allow for the dissipation of the native gas produced during the ablation process. Acquisition of 3D data set is carried out during the hepatic arterial, portal venous and late phase. Absence of any intra-lesional enhancement or moving microbubbles is consistent with complete coagulation necrosis and is easily depicted in hypervascular tumors. Residual viable tumor tissue is suspected when a portion of the original lesion maintains its micro-vascularity during the vascular phases. In enabling immediate further RFA of the residual disease, unnecessary delay may be avoided ensuring complete treatment within a single session. However, non-visualization of vascularity is not always a reliable indicator in the case of hypovascular

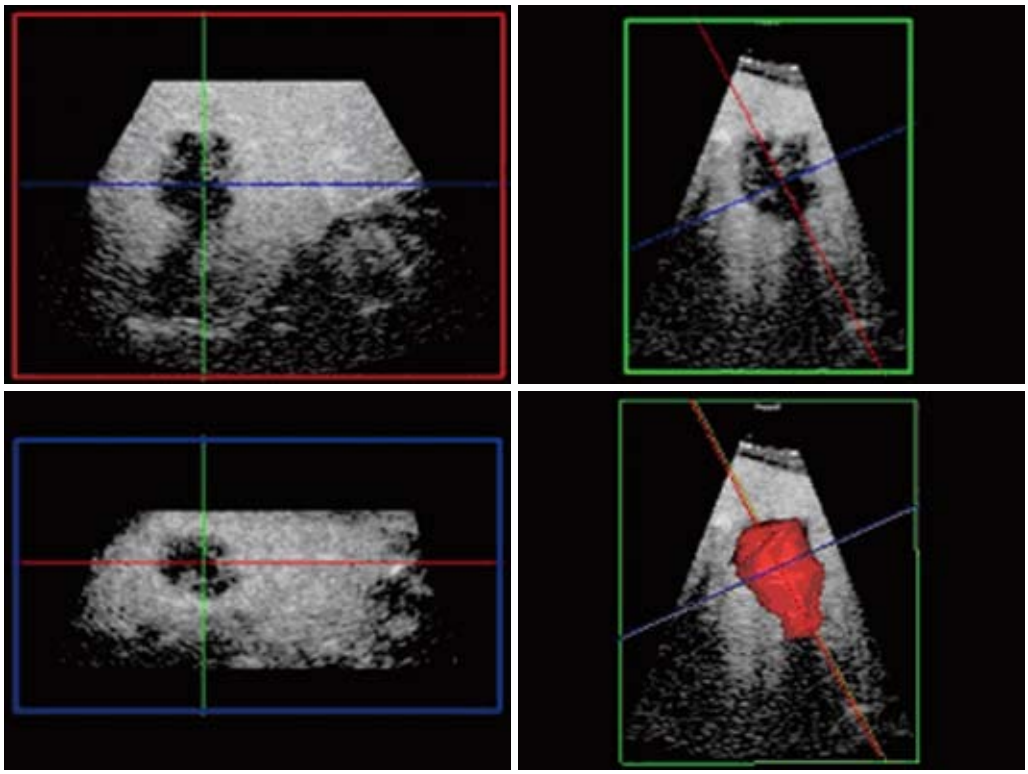

Figure 8 Multi-planar reconstruction. RFA Needle electrode placement along the long axis of the tumour mass which correspond to the planned "Red" axial plane bisecting the perpendicular the "Green" sagittal plane with reference to the probe position.

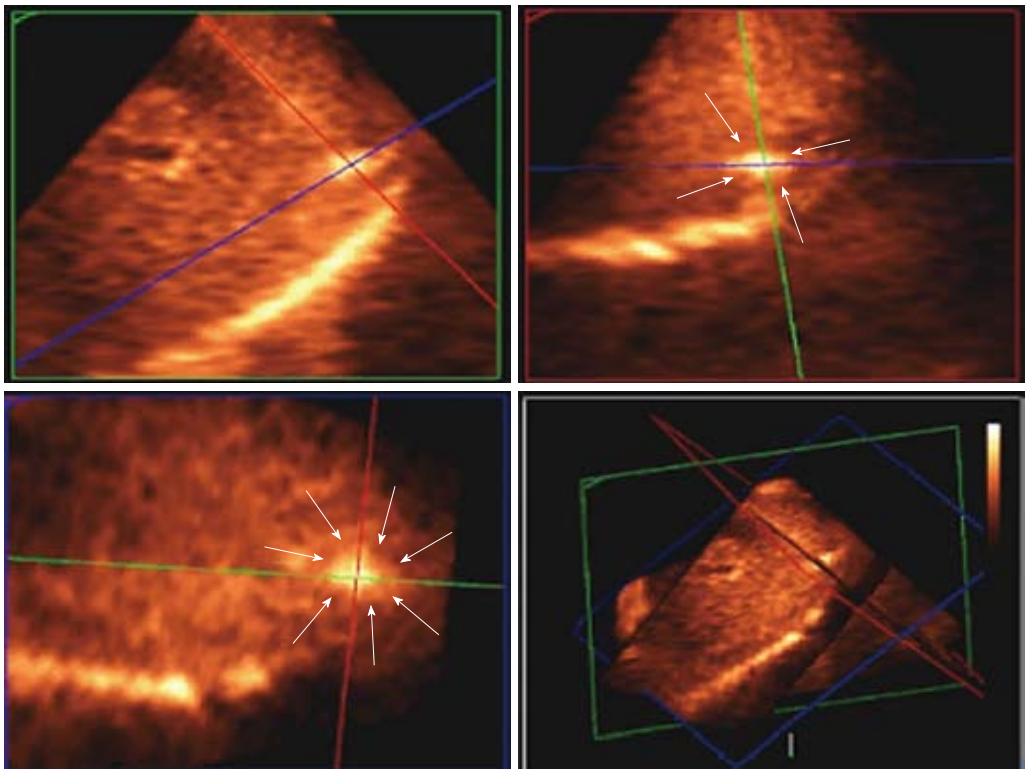

Figure 9 Matrix probe. 3D US showing the hyperechoic needle electrode tip and its spatial position in the tumour (white arrows delineates the tumour margin) in the subcapsular area and is clearly depicted in 3 orthogonal 2D images confirming the tip being in the center of the tumour.

tumors as in the case of most colorectal liver metastases. Complete coagulation necrosis may then be assessed through the side-by-side comparison of pre-RFA lesion size, volume and location, with those of the post-treatment coagulation necrosis. Peri-lesional "safety" margin adequacy must be evaluated at the same time. Clearly the advantage of CE-3DUS data acquisition during the vascular (arterial and portal) and late phases is evident to ascertain complete assessment of the volume of coagulation necrosis or presence of residual disease (Figure 12). Evaluation of the post RFA ablation volume relative to the pre RFA tumor volume may also be a particular additional important parameter in the assessment of response. A similar technique can also be used in the surveillance of these patients in the post ablative periods at 3, 6 or 12 mo; whilst this may not be optimal for patients with metastases who would benefit from CT in the surveillance for extra-hepatic disease,

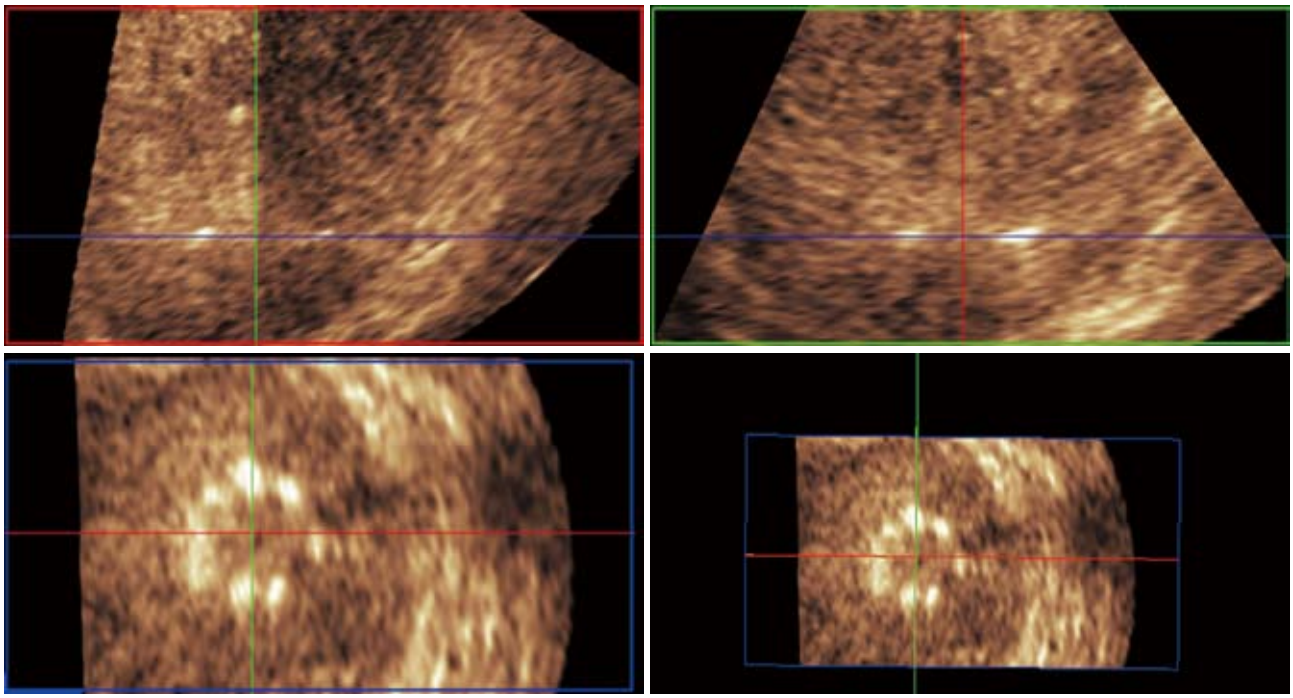

Figure 10 The 3 orthogonal planes showing the RFA needle electrodes with the multiple antennas deployed and clearly visualised on the (Blue: left lower quadrant) elevation plane following alignment of the Green and Red planes along the axis of the RFA needle electrode.

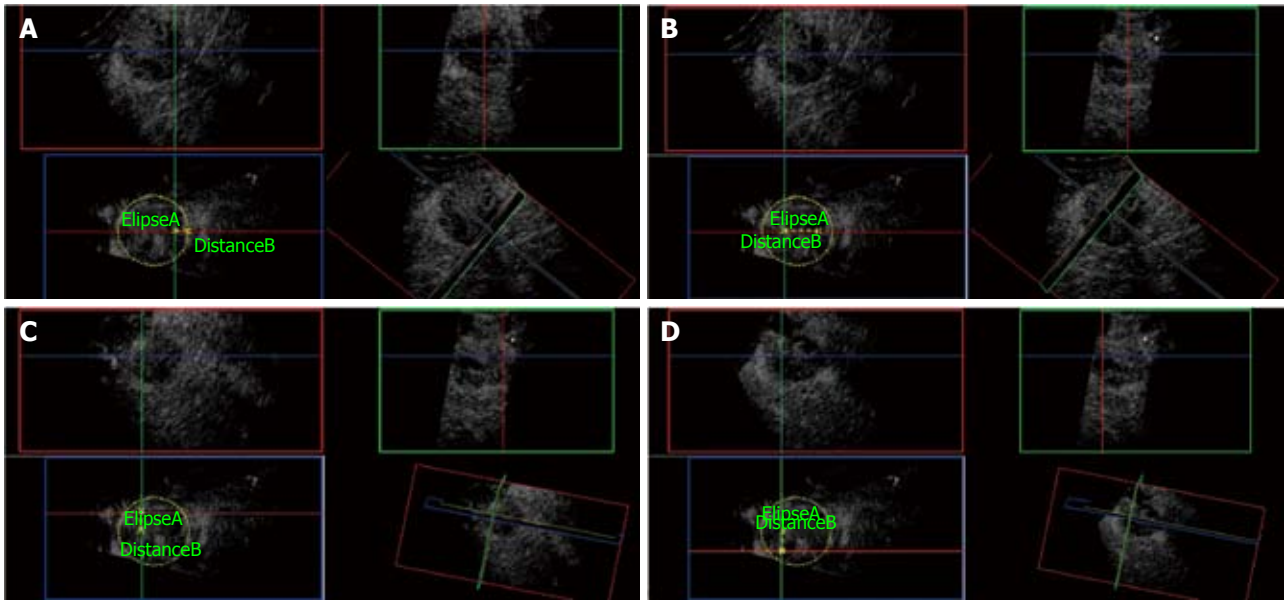

Figure 11 3D CE-US. A: Ellipse marks the tumor border at its maximal diameter (5 cm). First needle electrode insertion is planned from the elevation plane (Blue box) at 1 cm from the edge of the tumor. Green and Red orthogonal planes mark the trajectory of the needle electrode. B: Distance B measures 2.5 cm from the first needle electrode insertion and plans the plane (Green) of insertion for the next two needle electrodes. C: The second needle electrode is placed at the Green and Red planes intersection measuring 1.5 cm superior to the last position. D: Third needle insertion is placed at the Red and Green plane intersection, 3 cm below the second needle electrode as shown on the Blue plane.

it may be adequate for the surveillance of patients with hepatocellular carcinomas due to the much lower incidence of extra-hepatic dissemination.

Once local recurrence has been identified, CE-3DUS evaluation in the arterial phase and multi-planar reconstruction modelling are important in the planning for further RFA in that the geometry of the mass is usually non-spherical (Figure 13).

LIMITATIONS OF CE-3DUS

The spatial resolution of the current 3D probes is still limited on the non-linear imaging modes such that border delineation on the elevation plane is relatively poor. Volumetric acquisition of the data may be associated with distortion of the volume as a result of motion when using mechanical probes; however, this is not evident

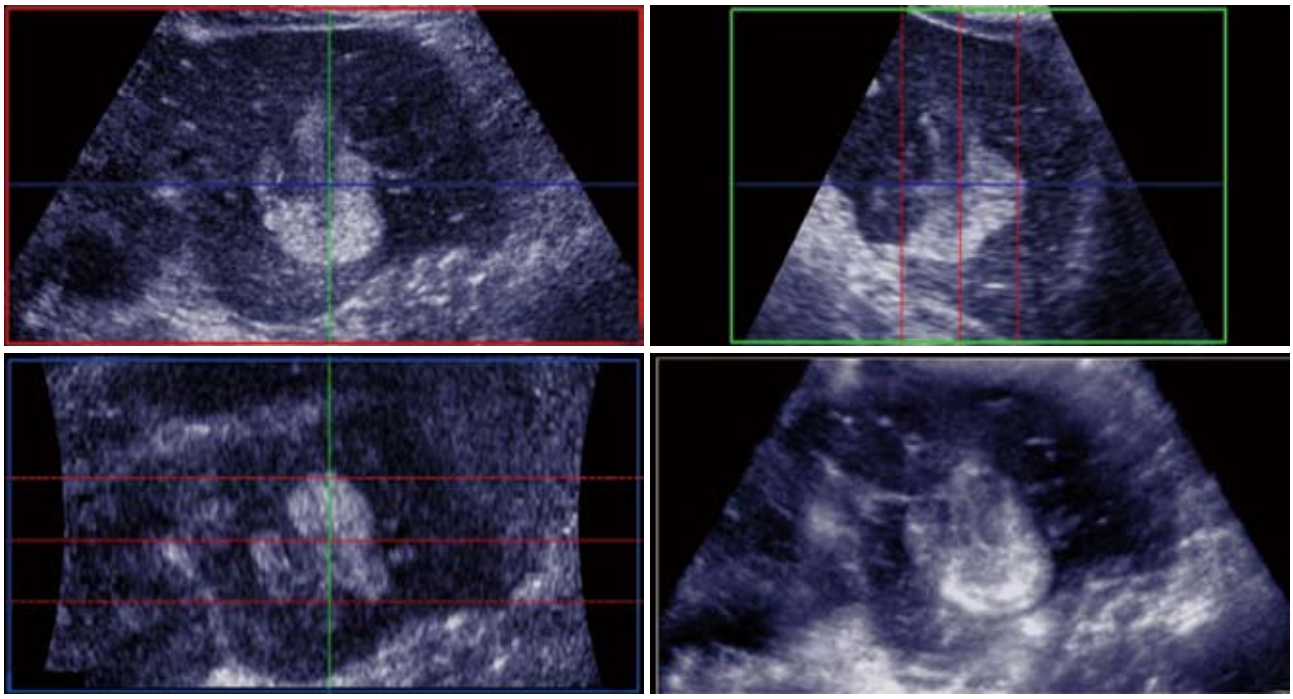

Figure 12 3D CE-US. Arterial phase 3D acquisition showing recurrent enhancing HCC in 3 orthogonal planes and the volume rendering analysis (left lower quadrant).

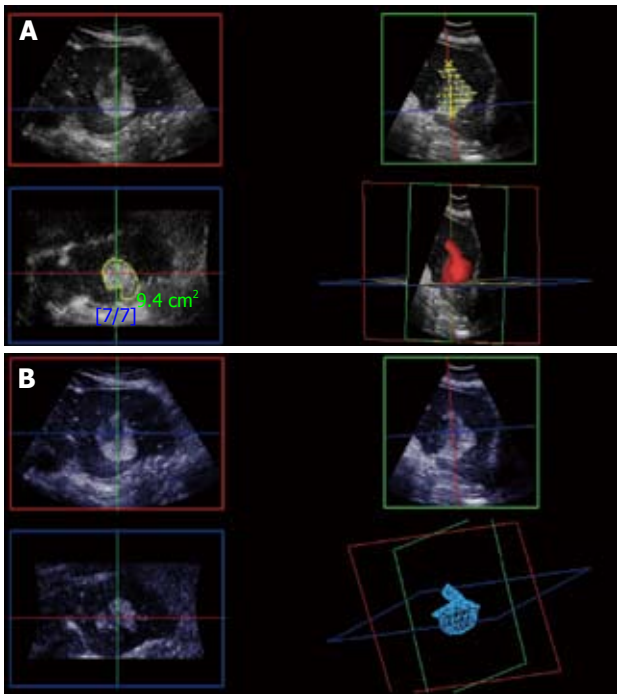

Figure 13 3D reconstruction of local recurrence geometry in the planning for further ablation. A: With normal liver background; B: Without normal liver background.

with the new electronic matrix probes largely due to the latter higher speed of volumetric scanning. The presence of native gas produced during the ablative process may produce shadowing artifacts, which may lead to inaccurate assessment of treatment response and calculation of ablation zone dimensions. There is a significant learning curve in the adoption of the new technique especially in the manipulation of the orthogonal planes.

FUTURE

One can expect further improvement in the spatial and temporal resolution of future 3D probes which would further facilitate live CE-3DUS targeting of the liver tumors in all three orthogonal planes simultaneously and enable real-time evaluation of the RFA needle electrodes alignment within the tumor volume on the elevation plane. Tissue perfusion is one of the functional parameters which can be used to assess tissue viability and may be used to assess response to treatment as well as a prognostic indicator. Whilst tissue perfusion quantification can be assessed on CE 2D-US, it is only valid if there is absolutely no movement during the data acquisition. Perfusion assessment along one single plane scan is also not representative of the whole tumor mass perfusion. Clearly 3D perfusion quantification is urgently needed for accurate functional imaging which may be incorporated into routine clinical practice through parametric imaging.

CONCLUSION

CE-3DUS is useful in the radiofrequency ablation (RFA) of liver tumors with improved detection and display of occult lesions and recurrence, in the assessment of lesional geometry and orientation for a more accurate planning and guidance of multiple RFA needles electrodes in large tumors and in the evaluation of residual or recurrent disease within the immediate and/or subsequent follow-up periods.

REFERENCES

1 **O'Brien MJ**. Cancer of the colon and rectum: current

concepts of aetiology and pathogenesis. *Ir J Med Sci* 1988; **157**: 5-15
2 **McArdle CS**, Hole D, Hansell D, Blumgart LH, Wood CB. Prospective study of colorectal cancer in the west of Scotland: 10-year follow-up. *Br J Surg* 1990; **77**: 280-282
3 **Scheele J**, Stang R, Altendorf-Hofmann A, Paul M. Resection of colorectal liver metastases. *World J Surg* 1995; **19**: 59-71
4 **Fong Y**, Cohen AM, Fortner JG, Enker WE, Turnbull AD, Coit DG, Marrero AM, Prasad M, Blumgart LH, Brennan MF. Liver resection for colorectal metastases. *J Clin Oncol* 1997; **15**: 938-946
5 **Adam R**, Bismuth H, Castaing D, Waechter F, Navarro F, Abascal A, Majno P, Engerran L. Repeat hepatectomy for colorectal liver metastases. *Ann Surg* 1997; **225**: 51-60; discussion 60-62
6 **Fernández-Trigo V**, Shamsa F, Sugarbaker PH. Repeat liver resections from colorectal metastasis. Repeat Hepatic Metastases Registry. *Surgery* 1995; **117**: 296-304
7 **El-Serag HB**, Mason AC. Risk factors for the rising rates of primary liver cancer in the United States. *Arch Intern Med* 2000; **160**: 3227-3230
8 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430
9 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699
10 **d'Arsonval MA**. Action physiologique des courants alternatifs. *CR Soc Biol* 1891; **43**: 283-286
11 **Lin SM**, Lin CJ, Lin CC, Hsu CW, Chen YC. Randomised controlled trial comparing percutaneous radiofrequency thermal ablation, percutaneous ethanol injection, and percutaneous acetic acid injection to treat hepatocellular carcinoma of 3 cm or less. *Gut* 2005; **54**: 1151-1156
12 **Berber E**, Pelley R, Siperstein AE. Predictors of survival after radiofrequency thermal ablation of colorectal cancer metastases to the liver: a prospective study. *J Clin Oncol* 2005; **23**: 1358-1364
13 **Curley SA**. Radiofrequency ablation of malignant liver tumors. *Ann Surg Oncol* 2003; **10**: 338-347
14 **Goldberg SN**, Gazelle GS, Solbiati L, Livraghi T, Tanabe KK, Hahn PF, Mueller PR. Ablation of liver tumors using percutaneous RF therapy. *AJR Am J Roentgenol* 1998; **170**: 1023-1028
15 **Lencioni R**, Goletti O, Armillotta N, Paolicchi A, Moretti M, Cioni D, Donati F, Cicorelli A, Ricci S, Carrai M, Conte PF, Cavina E, Bartolozzi C. Radio-frequency thermal ablation of liver metastases with a cooled-tip electrode needle: results of a pilot clinical trial. *Eur Radiol* 1998; **8**: 1205-1211
16 **Tournigand C**, André T, Achille E, Lledo G, Flesh M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C, de Gramont A. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J Clin Oncol* 2004; **22**: 229-237
17 **Kooby DA**, Fong Y, Suriawinata A, Gonen M, Allen PJ, Klimstra DS, DeMatteo RP, D'Angelica M, Blumgart LH, Jarnagin WR. Impact of steatosis on perioperative outcome following hepatic resection. *J Gastrointest Surg* 2003; **7**: 1034-1044
18 **Vauthey JN**, Pawlik TM, Ribero D, Wu TT, Zorzi D, Hoff PM, Xiong HQ, Eng C, Lauwers GY, Mino-Kenudson M, Risio M, Muratore A, Capussotti L, Curley SA, Abdalla EK. Chemotherapy regimen predicts steatohepatitis and an increase in 90-day mortality after surgery for hepatic colorectal metastases. *J Clin Oncol* 2006; **24**: 2065-2072
19 **Moug SJ**, Leen E, Horgan PG, Imrie CW. Radiofrequency ablation has a valuable therapeutic role in metastatic VIPoma. *Pancreatology* 2006; **6**: 155-159
20 **Conlon R**, Jacobs M, Dasgupta D, Lodge JP. The value of intraoperative ultrasound during hepatic resection compared with improved preoperative magnetic resonance imaging. *Eur J Ultrasound* 2003; **16**: 211-216
21 **Jarnagin WR**, Bach AM, Winston CB, Hann LE, Heffernan N, Loumeau T, DeMatteo RP, Fong Y, Blumgart LH. What is the yield of intraoperative ultrasonography during partial hepatectomy for malignant disease? *J Am Coll Surg* 2001; **192**: 577-583
22 **Cervone A**, Sardi A, Conaway GL. Intraoperative ultrasound (IOUS) is essential in the management of metastatic colorectal liver lesions. *Am Surg* 2000; **66**: 611-615
23 **Charnley RM**, Morris DL, Dennison AR, Amar SS, Hardcastle JD. Detection of colorectal liver metastases using intraoperative ultrasonography. *Br J Surg* 1991; **78**: 45-48
24 **Leen E**, Horgan P. Ultrasound contrast agents for hepatic imaging with nonlinear modes. *Curr Probl Diagn Radiol* 2003; **32**: 66-87
25 **Wilson SR**, Burns PN. Liver mass evaluation with ultrasound: the impact of microbubble contrast agents and pulse inversion imaging. *Semin Liver Dis* 2001; **21**: 147-159
26 **Albrecht T**, Blomley MJ, Burns PN, Wilson S, Harvey CJ, Leen E, Claudon M, Calliada F, Correas JM, LaFortune M, Campani R, Hoffmann CW, Cosgrove DO, LeFevre F. Improved detection of hepatic metastases with pulse-inversion US during the liver-specific phase of SHU 508A: multicenter study. *Radiology* 2003; **227**: 361-370
27 **Leen E**, Ceccotti P, Moug SJ, Glen P, MacQuarrie J, Angerson WJ, Albrecht T, Hohmann J, Oldenburg A, Ritz JP, Horgan PG. Potential value of contrast-enhanced intraoperative ultrasonography during partial hepatectomy for metastases: an essential investigation before resection? *Ann Surg* 2006; **243**: 236-240
28 **Leen E**, Moug S, Logue J, Darrien J, Angerson WJ, Horgan P. Value of contrast enhanced ultrasound (CE-US) in the radiofrequency ablation (RFA) of focal liver tumours: is it needed? *Radiology* 2005: Supplement 2005
29 **Bricault I**, Kikinis R, Morrison PR, Vansonnenberg E, Tuncali K, Silverman SG. Liver metastases: 3D shape-based analysis of CT scans for detection of local recurrence after radiofrequency ablation. *Radiology* 2006; **241**: 243-250
30 **Downey DB**, Fenster A, Williams JC. Clinical utility of three-dimensional US. *Radiographics* 2000; **20**: 559-571
31 **Xu HX**, Yin XY, Lu MD, Xie XY, Xu ZF, Liu GJ. Usefulness of three-dimensional sonography in procedures of ablation for liver cancers: initial experience. *J Ultrasound Med* 2003; **22**: 1239-1247
32 **Rose SC**, Hassanein TI, Easter DW, Gamagami RA, Bouvet M, Pretorius DH, Nelson TR, Kinney TB, James GM. Value of three-dimensional US for optimizing guidance for ablating focal liver tumors. *J Vasc Interv Radiol* 2001; **12**: 507-515

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ORIGINAL ARTICLES

Transient and etiology-related transcription regulation in cirrhosis prior to hepatocellular carcinoma occurrence

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Abstract

AIM: To search for transcription dysregulation that could (1) differentiate hepatocellular carcinoma (HCC)-free from HCC-related cirrhosis (2) differentiate HCC-free cirrhosis related to HCV from that related to alcohol intake.

METHODS: Using microarray analysis, we compared transcript levels in HCC-free cirrhosis (alcoholism: 7; hepatitis C: 7), HCC-associated cirrhosis (alcoholism: 10; hepatitis C: 10) and eight control livers. The identified transcripts were validated by qRT-PCR in an independent cohort of 45 samples (20 HCC-free cirrhosis; 15 HCC-associated cirrhosis and 10 control livers). We also confirmed our results by immunohistochemistry.

RESULTS: In HCC-free livers, we identified 70 transcripts which differentiated between alcoholic-related cirrhosis, HCV-related cirrhosis and control livers. They mainly corresponded to down-regulation. Dysregulation of Signal Transduction and Activator of Transcription-3 (STAT-3) was found along with related changes in STAT-3 targets which occurred in an etiology-dependent fashion in HCC-free cirrhosis. In contrast, in HCC, such transcription dysregulations were not observed.

CONCLUSION: We report that transcriptional dysregulations exist in HCC-free cirrhosis, are transiently observed prior to detectable HCC onset and may be appear like markers from cirrhosis to HCC transition.

Key words: Liver; Pathology; Alcoholism; Hepatitis C virus; Gene expression; Carcinogenesis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prominent, primary liver cancer. Its main etiologies are viral hepatitis B or C (HBV; HCV), alcoholism or aflatoxin B1 intoxication, with both HCV and alcoholism currently increasing in incidence in Western countries and predominating as etiologies[1,2]. In most instances, HCC develops in the setting of chronic hepatitis and/or cirrhosis[3]. Cirrhosis is the end stage of a chronic liver disease which results in regenerating nodules surrounding by fibrous septa and, ultimately, may lead to cancerous nodules. HCC has a poor prognosis but,

apart from surgery, no major improvements in disease therapies have been recently reported[4], most likely because the heterogeneity of the disease and its various etiologies prevent any progress in our understanding of HCC development and mechanisms[5].

Numerous genome-wide analyses of abnormal gene expression in HCC as compared to normal, control livers, have resulted in identification of gene sets with altered expression[2,6,7], part of which result from underlying gene mutations and/or chromosome alterations[8-10] and account for a limited number of altered pathways[9,11]. A few similar studies have been done in HCC-free cirrhosis and they mostly considered markers for a pre-HCC condition[12], or selected pathways[13], or mixed etiologies[12,14]. In fact, the number of comparative studies devoted to HCC etiology has remained scarce, whether this was done in a clinical setting[15-19], cell lines[20] or animal models with oncogene overexpression[21]. In particular, the viral etiologies have been considered[17,18,20,22] whereas abnormal gene expression in alcoholism-dependent HCC has received very little attention. Therefore, the impact of etiology still remains an important issue[7,23]. We recently reported that a number of genome-wide abnormalities in alcoholism-associated *vs* HCV-associated HCC are etiology-dependent and some of them are of pathological relevance[24]. Remarkably, the abnormal transcription levels that differentiate HCC nodules in an alcoholism-dependent *vs* HCV-dependent fashion can no longer discriminate between both etiologies when transcripts are measured in the surrounding cirrhosis[24]. Yet, any etiology-dependent abnormalities that could be observed in HCC-free cirrhosis would be of interest. We investigated whether some transcription dysregulations could be found in HCC-free cirrhosis in an etiology-dependent fashion. Furthermore, we searched and found transcript dysregulations that differentiate HCC-free cirrhosis from peritumoral cirrhosis. We now report that such transcription dysregulations do exist in HCC-free cirrhosis and are observed prior to detectable HCC onset.

MATERIALS AND METHODS

Human subjects and tissue sampling

Non-alcoholic steatohepatitis, primary biliary cirrhosis and infant biliary atresia were excluded from this study. Chronic alcohol abuse was estimated as detailed[24]. HBV and HCV infections were serologically determined in every patient and any HBV-positive patient was excluded. Patients with an HCC-free or HCC-associated cirrhosis were histologically diagnosed by trained pathologists (AF, PBS). Liver fragments came to our laboratory from the digestive surgery unit of Charles Nicolle Hospital (Rouen, France) or the pathology unit of Pellegrin Hospital (Bordeaux, France) under strict anonymity. HCC-free, cirrhotic tissue was obtained from transplanted patients. Peri-tumoral, cirrhotic tissue was taken at a distance from HCC resection whenever the latter was excised for curative purposes. Control, non-cirrhotic human liver (CL) was obtained from patients operated on for a benign liver tumor or metastasis of a non-hepatic cancer. According to the current French rules and ethical guidelines, neither informed consent nor advice from an ethical committee were requested prior to RNA analysis in tissues that would otherwise be disposed of. Various clinical features in a total of 69 cirrhosis without HCC, (n = 34) or with HCC (n = 35), as well as in a set of 18 histologically normal CLs are summarized in Table 1. A METAVIR score from the combined extents of inflammation (A0-A3) and fibrosis (F0-F4) were histologically diagnosed by trained pathologists (AF, PBS).

Transcriptome analysis and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA extraction from tissues stored at -80℃ was done with Trizol. Our set of human cDNA probes dubbed *Liverpool* and tailored to a complete coverage of the human liver transcriptome under healthy or pathological conditions (ca. 10^4 genes), the associated *LiverTools* database, as well as the procedures from array preparation to data handling have all been detailed[25]. In brief, every RNA sample was subjected to three rounds of hybridization and the resulting signals were normalized from the average signal of every spot (mean grey) on the matching hybridization image. The mean signal per transcript was used for selections of significantly regulated transcripts. Probe re-sequencing was done with an ABI3100 capillary sequencer (Applied Biosystems, Foster City, USA). Real-time qRT-PCRs of transcripts were done with a Light Cycler (Roche Diagnostics, Manheim, Germany). Transcript normalization was done with the 18S RNA. The primers designed with the Primer3 software (http://frodo.wi.mit.edu) are listed in a Table 2.

Data mining

Our raw data are deposited in the GEO repository under accession number GSE10356. The TIGR Multiexperiment viewer (Tmev version 2.2, http://www.tm4.org) was used for (1) unsupervised hierarchical clustering (UHC) using the Manhattan distance and complete linkage options; (2) supervised analyses such as the *t*-test or ANOVA adjusted with Bonferroni's correction or K-nearest neighbour classification (KNNC) and (3) evaluation of sample re-assignment by a random procedure (jackknife-10^6 iterations). Another, supervised classification was done by Support Vector Machine (SVM) (http://svm.sdsc.edu/). The Gene Ontology Tree Machine (GOTM) program (http://bioinfo.vanderbilt.edu/gotm/) was used to categorize protein function(s) by ontology. Detailed protein functions were retrieved with the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and/or OMIM (http://www.ncbi.nlm.nih.gov/sites/entrez) tools. Protein networks were identified with Bibliosphere (www.genomatix.de). Statistics were carried out with the GraphPad Instat software, version 3 (http://www.graphpad.com/).

Table 1 Biological and clinical data from patients with cirrhosis alone, HCC-associated cirrhosis and controls

Patient[1]	Number	Male/Female	Age[2]	Pathology[3]	Etiology[4]	Metavir[5]			
						A0	A1	A2	A3
A1 to A7	7	3/4	48.4 ± 3.2	CIR	ALC	0	4	1	2
A15 to A24	10	7/3	50.4 ± 7.5	CIR	ALC	0	8	2	0
PA1 to PA10	10	8/2	67.1 ± 8.0	CIR + HCC	ALC	2	5	3	0
PA21 to PA29	9	9/0	60.9 ± 8.2	CIR + HCC	ALC	1	7	1	0
V8 to V14	7	6/1	57.4 ± 8.9	CIR	HCV	0	2	4	1
V25 to V34	10	5/5	55.7 ± 16.0	CIR	HCV	0	5	3	2
PV11 to PV20	10	6/4	71.5 ± 5.1	CIR + HCC	HCV	0	1	6	3
PV30 to PV35	6	2/4	66.2 ± 9.5	CIR + HCC	HCV	0	1	3	2
CL1 to CL8	8	3/5	59.9 ± 15.9	No CIR, no HCC[6]	--	7	1	0	0
CL9 to CL18	10	2/8	49.5 ± 14.0	No CIR, no HCC[6]	--	9	1	0	0

[1]HCC-free alcoholic cirrhosis; PA: Peritumoral alcoholic cirrhosis; V: HCC-free HCV cirrhosis; PV: Peritumoral HCV cirrhosis; CL: Control without any detectable fibrosis. Underlined samples were studied by microarray and qRT-PCR; No underlining, independent cohort of samples studied by qRT-PCR only. [2]mean ± SD. [3]CIR: Cirrhosis; CIR + HCC: Peritumoral cirrhosis. [4]HCV: Hepatitis C virus infection; ALC: Alcoholism; --: None. [5]From left to right, number of patients with a given score of inflammation A0-A3. Difference in METAVIR score in HCC-free cirrhosis between all V *vs* A patients, $P = 0.17$ (Mann and Whitney's test); In peritumoral cirrhosis between all PV *vs* PA patients, $P = 0.005$. [6]Histologically normal liver sampled at a distance from a benign liver tumor or from a metastasis of non-hepatic cancer.

Table 2 Oligonucleotides for qRT-PCR

Oligonucleotides	Forward	Reverse	Amplicon size (bp)
ACSM2	AAATCCCGACAAGACAGCAG	CTGATCACAGCCGTCTCAAC	201
GSTA1/2	TCTGCAGAAGATTTGGACAAG	TCAATCAGGGCTCTCTCCTT	170
ADH4	GTCTGCTTGGATGTGGGTTT	TGATTCTGGAAGCTCCTGCT	150
HSCARG	GAAACTGGTGGTGGTTTTCG	CATCTTGGTCTCCCTGCACT	170
AHNAK	CAAAGGGAAACACACCGACT	GCTCTCAGCAGTCAATGCAA	207
HSD17B6	TCTGGGGACTGGTGAACAAT	GTGCTCTCCTCACCAAAGGA	150
APOH	CCGAGGAGGGATGAGAAAGT	AGAATCAGCGCCATTCAGAT	193
IFI27	CCAAGCTTAAGACGGTGAGG	AAAACTACGGCAGAGCCAGA	196
ARID1A	CTACGCTGCCACGTGTGTAT	GTACAGCATCGCACCAAGAG	187
MT1G	TCCTGCAAGTGCAAAGAGTG	ACTTCTCCGATGCCCCTTT	118
ARL2BP	AGGATGAAGTGGCTGGTGAC	GGAAGCTGGCAGAGAAGATG	170
ORM2	TTATATCGCATCGGCCTTTC	CCGCTGGACATTCAGGTAAC	172
ATP5G2	TTGTCTCCACTCCCTCCTTG	TGTGTCGATGTCCCTTGAAA	191
PLG	GTAGGTGGTCCCTGGTGCTA	CCTACAACCCTTCCAGGACA	137
CYP3A4	CTTTGGAAGTGGACCCAGAA	CGGGTTTTTCTGGTTGAAGA	164
STAT3	CCCCATACCTGAAGACCAAG	CTCCGAGGTCAACTCCATGT	185
CYP2E1	TCAAGCCATTTTCCACAGGA	CGATATCCTTTGGGTCAACGA	129
TIMP1	AATTCCGACCTCGTCATCAG	GTTGTGGGACCTGTGGAAGT	195
DPF2	CTCCTGGCTCACTCTTACGG	AAGGGGATTTTGGAGGTAGG	211
18S	GTGGAGCGATTTGTCTGGTT	CGCTGAGCCAGTCAGTGTAG	200
DPM1	GCAGTCCACGACAGAACAAA	CATCTGGGCTTCCATCATCT	150

Immunohistochemistry

D4 zinc and double PHD fingers family 2 (DPF2) and plasminogen (PLG) protein levels were assessed in formaldehyde-fixed, paraffin-embedded, 5 mm thick liver section samples giving a total of 40 other cirrhosis without or with HCC (10 alcoholic and 10 HCV in each group), as well as a set of 10 other histologically normal CLs. This assessment was effected by immunohistochemistry using the ultraView™ Universal DAB Detection Kit following the manufacturers instructions (Ventana Medical systems) with mouse anti-DPF2 IgGs (Abnova corporation) at 1 μg/mL or mouse anti-PLG IgGs (Interchim) at 38 μg/mL.

The percentage of positive cells was evaluated on the whole surface of the histological section and the staining intensity was estimated. Two scores from 0 to 4 were given as two independent visual scores by a trained pathologist and were evaluated using a LEICA DMR microscope equipped with a camera. The number of positive cells or P score was: 0, no positivity; 1, < 25%; 2, 25%-50%; 3, 50%-75% and 4, > 75%-100%. The determination of immunostaining intensity or I score was: 0, no staining; 1, very weak staining only seen at magnification × 10; 2, staining obviously seen at magnification × 10; 3, moderate staining seen at magnification × 2.5; 4, strong staining seen at magnification × 2.5. The IP score was obtained from the additional combination of the two parameters I + P.

RESULTS

Different transcriptome alterations in HCC-free or peritumoral cirrhosis vs CLs

First, with microarray data from 14 HCC-free cirrhosis samples (A1-A7, V8-V14) and eight CLs (CL1-CL8), we identified 30 transcripts

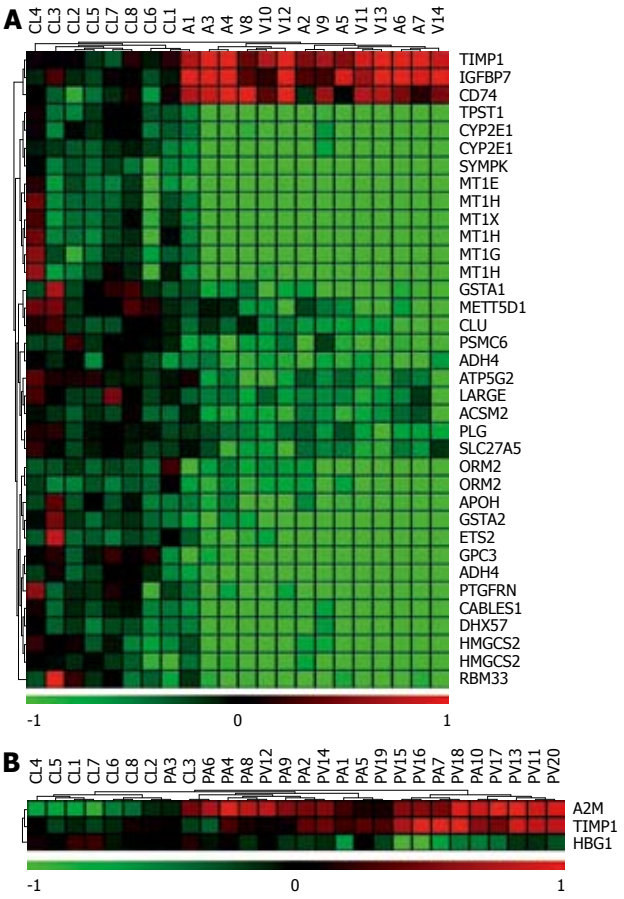

Figure 1 Clustering of cirrhosis from comparisons of transcript levels *vs* CIs. Every transcript level expressed as a [level per patient/median level in CLs] was measured by microarray. The samples are shown as a dendrogram on top and the transcripts are listed vertically. Bottom scale bar (log2 scale): decreased (green), increased (red) or unchanged (black) transcript level. A: UHC was made in 14 HCC-free cirrhosis samples and 8 CLs, from 30 transcript levels first identified as cirrhosis markers in an HCC-free context. B: UHC was made with 20 peritumoral cirrhosis samples and 8 CLs, from 3 transcript levels identified as cirrhosis markers in an HCC context.

whose levels differed between [A + V] cirrhosis *vs* CLs (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 1A). In contrast, similar data from 20 peritumoral cirrhosis samples (PA1-PA10, PV11-PV20) and eight CLs identified only three transcripts, but they did not distinguish [PA-PV] from CLs (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 1B). This HCC-dependent difference in transcript number was significant (30 *vs* 3, Fisher's test, $P < 0.0001$). Furthermore, the expression levels of the 30 transcripts, which distinguished HCC-free cirrhosis, from CLs, did not distinguish [PA-PV] from CLs (data not shown). Thus, we identified transcripts which were dysregulated in HCC-free cirrhosis but not in peritumoral cirrhosis.

Different transcriptome alterations in alcoholic- vs HCV cirrhosis vs CLs

Next, the comparison of transcript levels in alcoholic HCC-free cirrhosis *vs* CLs identified 10 dysregulated transcripts (*t*-test adjusted by Bonferroni's correction, $P < 0.05$). Likewise, we identified 49 dysregulated transcripts in HCV HCC-free cirrhosis *vs* CLs. We also found 33 transcripts that were differentially expressed when directly comparing alcoholic *vs* HCV HCC-free cirrhosis. Overall, we obtained a non-redundant list of 70 transcripts whose levels were able to completely distinguish between the three groups by UHC: alcoholic, HCV HCC-free cirrhosis and CLs (Figure 2A). This was supported by a jackknife procedure (100% success). In contrast, in HCC five transcripts failed to properly distinguish between these three groups (*t*-test adjusted by Bonferroni's correction, $P < 0.05$, Figure 2B). This HCC-related difference was significant (70 *vs* 5, Fisher's test, $P < 0.0001$). Furthermore, the expression levels of these 70 transcripts, which separated alcoholic, HCV and CLs in an HCC-free context, did not distinguish between them in an HCC context (data not shown).

Table 3 Performance of various, unsupervised or supervised classification tools for HCC-free cirrhosis samples

Samples[2]	Tool[1]		
	UHC (%)	SVM (%)	KNNC (%)
A1-A7 + A15-A24	65[3]	100	100
V8-V14 + V25-V34	71	80	70
CL1-CL18	100	70	80
All test samples	79	83	83

[1]Unsupervised hierarchical clustering (UHC) was done with 52 cirrhotic or CL samples. Supervised training/testing procedures (KNNC; SVM) were each done by first separating these 52 samples into 22 training (A1-A7, V8-V14 and CL1-CL8) and 30 test samples (A15-A24, V25-V34 and CL9-CL18). [2]20 transcript levels were measured in total RNA from every sample by qRT-PCR. These transcripts had the most significant difference in levels (Bonferroni-corrected ANOVA, $P < 0.05$) between alcoholism *vs* HCV *vs* CLs (ACSM2, ADH4, AHNAK, APOH, ARID1A, ARL2BP, ATP5G2, CYP2E1, CYP3A4, DPF2, DPM1, GSTA 1-2, HSCARG, HSD17B6, IFI27, MT1G, ORM2, PLG, STAT3, TIMP1). [3]% of properly classified test samples.

From our list of 70 transcripts obtained from our microarray data, we measured by real-time qRT-PCR the 20 most discriminant transcripts (as listed in Table 3, footnote 2) which were differentially expressed according to etiology. Using both the 22 training samples (A1-A7, V8-V14 and CL1-CL8) and 30 further independent test samples (A15-A24, V25-V34 and CL9-CL18) in order to classify HCC-free cirrhosis by unsupervised (UHC) or supervised training/testing procedures (KNNC and SVM),we found that these 20 transcripts resulted in a classification accuracy of 79%-83% test samples (Table 3).

Most transcriptome alterations in HCC-free cirrhosis are a transient event

The abnormalities in transcript levels seen in HCC-free cirrhosis, but not in peritumoral cirrhosis, were further evaluated timewise. As shown in Figure 3A (upper left star) the expression levels in CLs ($n = 18$), HCC-free cirrhosis ($n = 34$) and peritumoral cirrhosis ($n = 35$) were measured by qRT-PCR. When comparing the above 20 transcript levels between HCC-free *vs* CLs, their mean level in HCC-free cirrhosis was up-regulated (TIMP1), unchanged (3/20 transcripts, 15%, DPF2, IFI27, STAT3),

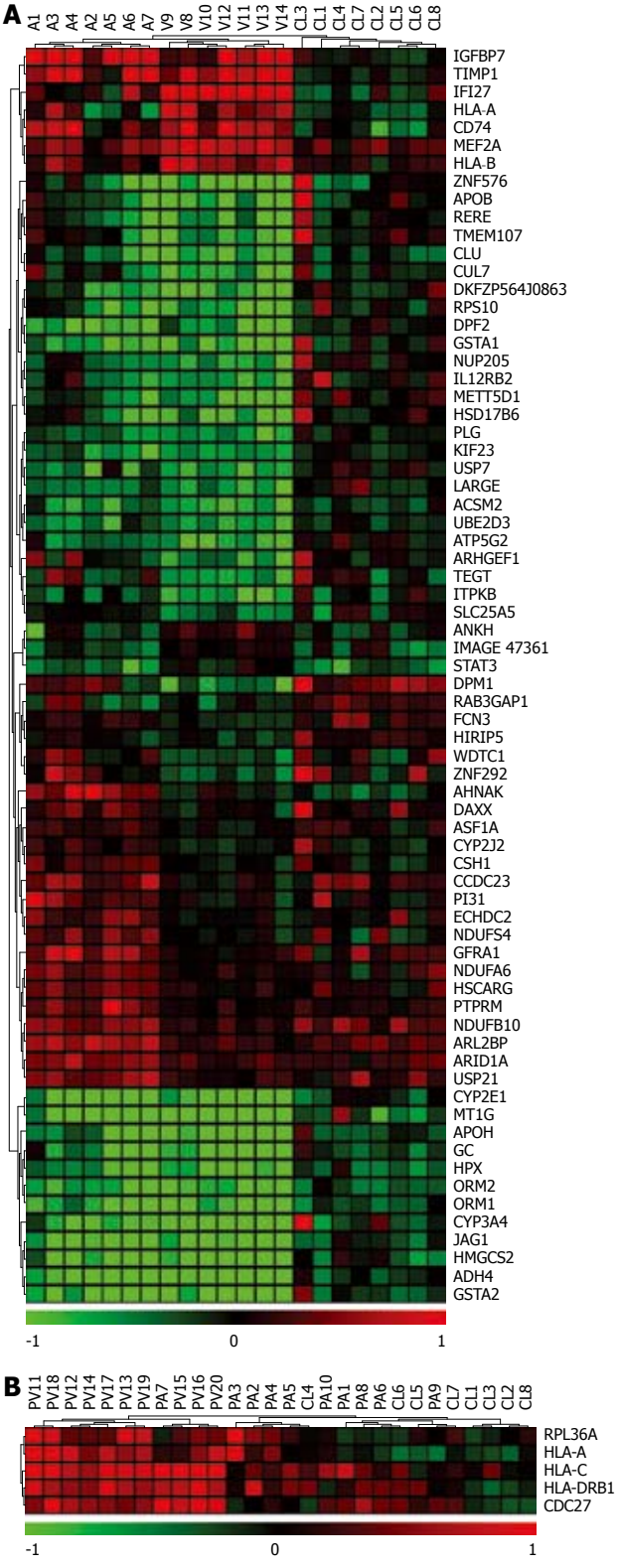

Figure 2 Clustering of cirrhosis samples from transcript levels. Every transcript was measured by microarray and expressed as a [level per patient/median level in CLs]. The samples are shown as a dendrogram on top and the transcripts are listed vertically. Bottom scale bar (log2 scale): decreased (green), increased (red) or unchanged (black) transcript level. A: UHC was made in 14 HCC-free cirrhosis samples and eight CLs, from 70 altered transcript levels first identified as markers of HCC-free cirrhosis. A or V, alcoholism-related or HCV-related etiology B: UHC was made in 20 HCC-associated cirrhosis samples and eight CLs, from five transcript levels identified by *t*-test adjusted by Bonferroni's correction. PA or PV, perinodular cirrhosis, with an alcoholism-related or HCV-related etiology, respectively.

or mostly down-regulated (16/20, 80%). In contrast, this down-regulation was not found when cirrhosis was associated with HCC. Indeed, in peri-tumoral cirrhosis a significant return to the CL level or even an up-regulation was observed (Figure 3A, upper right star). Moreover, as shown in Figure 3B, 9/20 (45%) transcript levels further displayed etiology-dependent differences found (1) only in HCC-free cirrhosis (alcoholism *vs* HCV, lower left star, 6/9 transcripts, 66%), or (2) only in peritumoral cirrhosis (lower right star, 2/9 transcripts, 22%, ARID1A, ORM2), or (3) in both (IFI27). Overall, these transcript dysregulations were mostly seen in HCC-free cirrhosis, often resulted from a transient down-regulation, and half of them were etiology-dependent in agreement with the initial selection.

Semi-quantitative immunodetection of DPF2 and PLG in liver samples

Among the nine genes mentioned above which displayed etiology-dependent differences, DPF2 and PLG, whose antibodies were marketed for immunohistochemistry use, were selected. We quantified their protein levels in a total of 40 other cirrhosis without or with HCC (10 alcoholic and 10 HCV in each group), as well as in a set of 10 other histologically normal CLs.

The DPF2 protein level was significantly higher in HCC-free cirrhosis, and then decreased in HCC-associated cirrhosis to return to a level similar to that observed in CLs, but this level regulation was mild (data not shown).

The PLG protein level was also significantly different in CLs, HCC-free and HCC-associated cirrhosis. Indeed, the PLG level was significantly lower in HCC-free cirrhosis as compared to that observed in CLs and then increased in HCC-associated cirrhosis to return to a level quite similar to that observed in CLs (Figure 4A). The immunohistochemical pattern for CLs with a strong hepatocellular staining was shown in Figure 4D). The PLG protein level also displayed etiology-dependent differences. Indeed, the decrease of the staining was significantly higher in HCC-free HCV cirrhosis (Figure 4E) than in HCC-free alcoholic cirrhosis (Figure 4B) and in HCC-associated cirrhosis the increase with a return to the baseline was the same whatever the etiology (HCV or alcohol) (Figure 4C and F). Thus, the PLG protein level confirmed our results obtained at the transcriptional level.

Functional differences in HCC-free vs peritumoral cirrhosis

We first investigated whether transcript variations could point to functional dysregulation in HCC-free cirrhosis. By comparing our list of 70 transcripts and our *Liverpool*, a different frequency of dysregulated transcripts in eight functional subsets was found (detailed as a Table 4): (1) cell proliferation ($P = 0.01$); (2) regulation of cell migration ($P = 0.009$); (3) blood vessel development ($P = 0.007$); (4) lipid metabolism ($P = 0.007$); (5) antigen processing and presentation

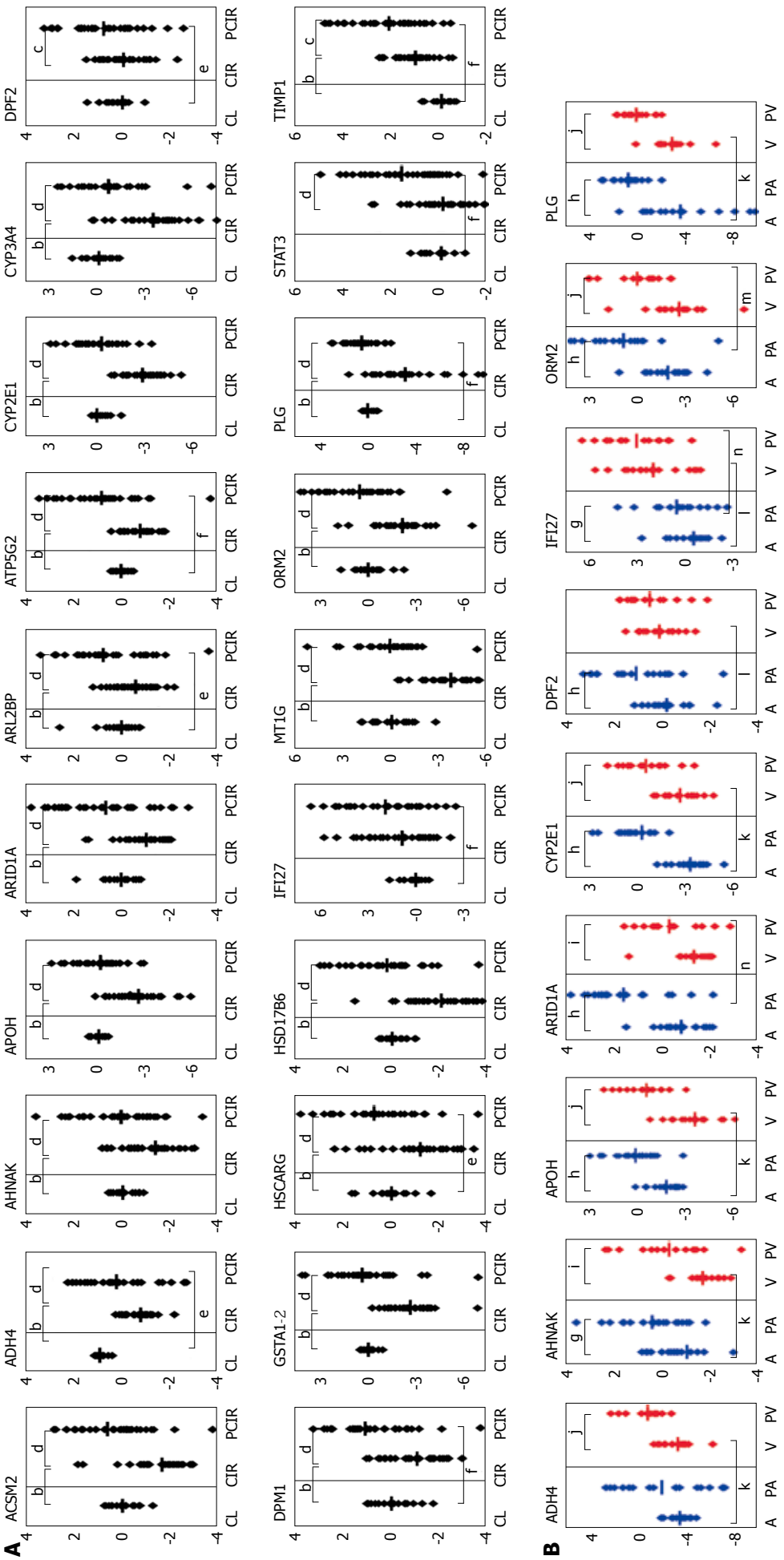

Figure 3 Changes in transcript levels in cirrhosis. The transcripts are those listed in Table 3, footnote 2F. Their levels were determined by real-time qRT-PCR in training and testing samples (18 CLs, 34 HCC-free cirrhosis and 35 peritumoral cirrhosis). Every transcript name is noted on top and its level per cirrhosis type is expressed on the ordinate as a log2 [level in type/median level in CLs]. The mean value of transcripts is shown as an horizontal bar. A : CL: control; CIR: HCC-free cirrhosis (regardless of etiology); PCIR: perinodular cirrhosis (regardless of etiology). Significant difference between CIR and CL ($^{a}P < 0.05$, $^{b}P < 0.01$, Mann-Whitney *U* test), between CIR and PCIR ($^{c}P < 0.05$, $^{d}P < 0.01$) and between PCIR and CL ($^{e}P < 0.05$, $^{f}P < 0.01$). B: Transcripts separated per etiology : alcoholism (blue), HCV (red); A or V: alcoholic or viral, HCC-free cirrhosis; PA or PV: perinodular, alcoholic or viral cirrhosis. Significant difference between A and PA ($^{g}P < 0.05$, $^{h}P < 0.01$), between V and PV ($^{i}P < 0.05$, $^{j}P < 0.01$), between A and V ($^{k}P < 0.05$, $^{l}P < 0.01$) and between PA and PV ($^{m}P < 0.05$, $^{n}P < 0.01$).

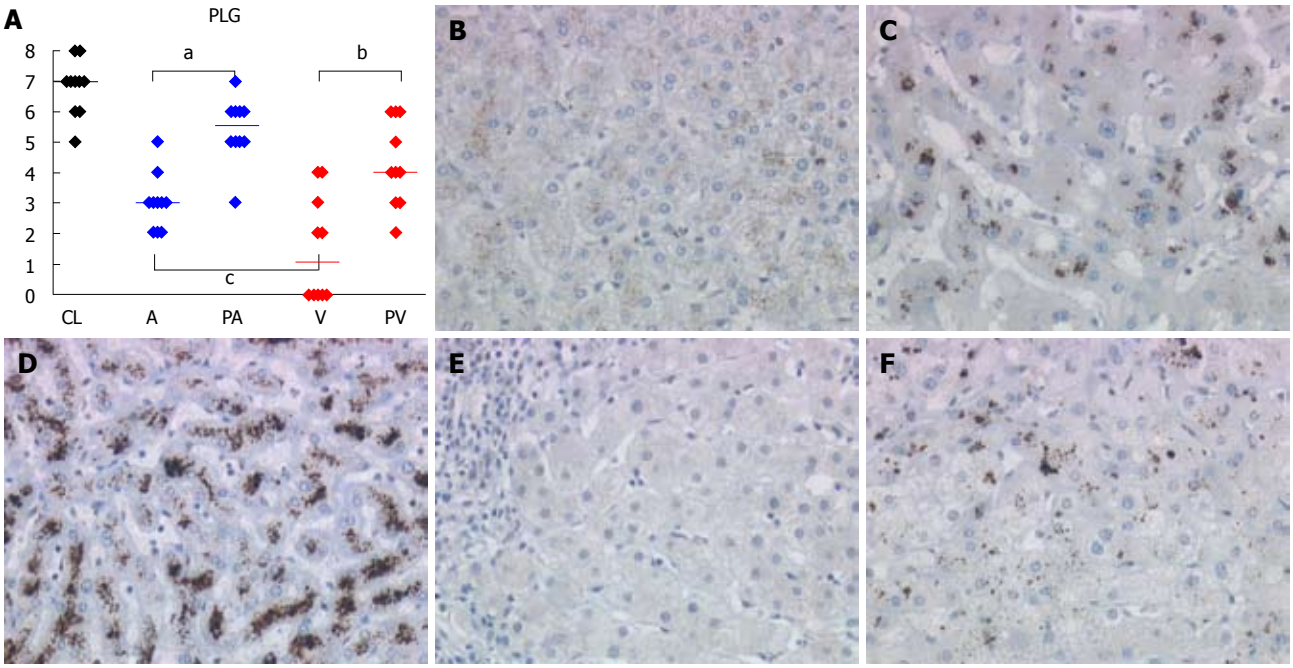

Figure 4 PLG protein expression in control liver, HCC-free and HCC-associated cirrhosis. The PLG protein levels were determined by immunohistochemistry (magnification x 20). A: Every protein level (IP score) per cirrhosis type is expressed on the ordinate. The mean value of protein level is shown as an horizontal bar. The samples abbreviations are the same as for Figure 3. Significant differences between A and PA ($^aP < 0.05$), between V and PV ($^bP < 0.05$), between A and V ($^cP < 0.05$). B-F: PLG immunostaining of hepatic sections corresponding to alcoholic HCC-free cirrhosis (B), alcoholic HCC-associated cirrhosis (C), control liver (D), HCV-related HCC-free cirrhosis (E) and HCV-related HCC-associated cirrhosis (F).

Table 4 Over-representation of functional subsets in our list of 70 transcripts

Cell proliferation[1] (O = 7; E = 2.66; *P* = 0.01[2])	Regulation of cell migration (O = 2; E = 0.15; *P* = 0.009)	Blood vessel development (O = 3; E = 0.41; *P* = 0.007)	Lipid metabolism (O = 9; E = 3.55; *P* = 0.007)	Antigen processing and presentation (O = 3; E = 0.25; *P* = 0.001)	Acute inflammatory response (O = 4; E = 0.58; *P* = 0.002)	NADH dehydrogenase activity (O = 3; E = 0.36; *P* = 0.005)	Oxidoreductase activity (O = 3; E = 0.25; *P* = 0.001)
JAG1 (Hs.590881[3])	JAG1 (Hs.590881)	JAG1 (Hs.590881)	CLU (Hs.436657)	HLA-A (Hs.181224)	CLU (Hs.436657)	NDUFA6 (Hs.274416)	CYP2E1 (Hs.12907)
IGFBP7 (Hs.479808)	PLG (Hs.143436)	PLG (Hs.143436)	CYP2J2 (Hs.152096)	HLA-B (Hs.77961)	ORM1 (Hs. 567311)	NDUFB10 (Hs.513266)	CYP2J2 (Hs.152096)
IL12RB2 (Hs.479347)		CUL7 (Hs.520136)	CYP3A4 (Hs.567254)	CD74 (Hs.591258)	ORM2 (Hs.522356)	NDUFS4 (Hs.528222)	CYP3A4 (Hs.567254)
PLG (Hs.143436)			HMGCS2 (Hs.59889)		STAT3 (Hs.463059)		
TIMP1 (Hs.522632)			APOB (Hs.120759)				
ARHGEF1 (Hs.438429)			HSD17B6 (Hs.524513)				
CD74 (Hs.591258)			DPM1 (Hs.301898)				
			LARGE (Hs.474667)				
			CD74 (Hs.591258)				

[1]This transcript subset coding for proteins with a related function was identified by gene ontology with the GOTM tool; [2]Significance of enrichment for the GO category between transcript number observed (O) and expected (E) in this cate gory; [3]Hs. number: Unique transcript identifier.

($P = 0.001$); (6) acute inflammatory response ($P = 0.002$); (7) NADH dehydrogenase activity ($P = 0.005$) and (8) oxidoreductase activity ($P = 0.001$).

Within our set of 70 transcripts, 23 transcripts with available information from Bibliosphere exhibited an etiology-associated level variation in HCC-free cirrhosis (Figure 5), but not in peritumoral cirrhosis (data not shown). This resulted from a variable extent of down-regulation (17/23 transcripts, 74%) in a single etiology or both. In turn, this down-regulation resulted, at least partly, from variable, etiology-dependent regulation of STAT-3 and its target genes (lower right area of Figure 5).

DISCUSSION

Our search first focused on the significance of

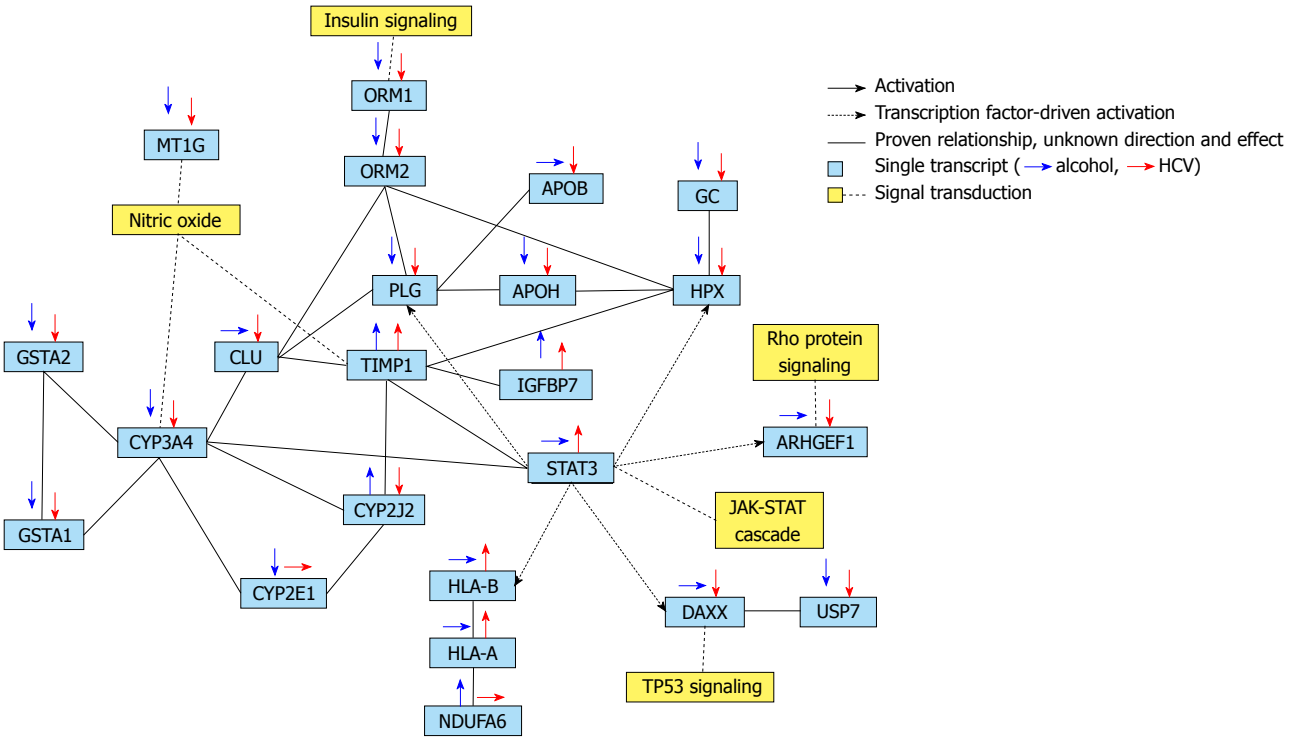

Figure 5 Networks of etiology-independent or dependent transcript regulations in HCC-free cirrhosis. From 70 transcripts with a significantly different expression between alcoholic-associated *vs* HCV-associated cirrhosis *vs* CLs ($P < 0.05$, as in Figure 3), 23 transcripts (boxes) associated with different pathways were identified in Bibliosphere. In a context of HCC-free cirrhosis, etiology-specific arrows (alcoholism, blue; HCV, red) above the transcript indicates the direction of regulation: upward, downward or horizontal arrow: up-regulation, down-regulation or unchanged regulation. For a transcript with an up- (down-) regulation in both etiologies, the highest (lowest) arrow indicates the most marked dysregulation.

dysregulations found in HCC-free cirrhosis. We investigated the influence of an HCC-free *vs* peritumoral environment of cirrhosis and we searched for early markers of HCC onset. The ideal study design would be to compare cirrhosis that develops into HCC and cirrhosis that does not develop into HCC in the follow-up of the disease, but such samples were very scarce because the follow-up among cirrhotic patients would be extremely long and difficult. So, we compared HCC-free and HCC-associated cirrhosis and we used a stringent selection of informative transcripts because sharp, timewise variations of potential markers of HCC occurrence were to be identified. We have shown that transcription dysregulation does exist in HCC-free cirrhosis. This is observed before any histologically detectable HCC nodule is seen, and hence supports the "field cancerization" model. Specifically, from our GOTM results, it seems plausible that malignant transformation of cirrhosis could be favored by abnormal expression of factors regulating cell migration, cell proliferation and blood vessel development. These dysregulations in cirrhosis mainly correspond to down-regulations, and they usually re-normalize or are even up-regulated in peritumoral cirrhosis. It remains to be determined how such transient down-regulations, which have been as yet unreported, contribute to HCC initiation.

We next carried out a similar search, further integrating alcoholism and HCV etiologies. A few, etiology-dependent markers for a high *vs* low risk of HCC development have been previously identified by others, but they relied on an unproven assignment of alcoholism or HCV patients to either risk group and they could not predict HCC occurrence in a timewise fashion[12]. We had previously reported that abnormalities of some transcript levels are observed to a different extent in HCC developed on alcoholic-associated cirrhosis *vs* HCV-associated cirrhosis, whereas they remain similar in peritumoral cirrhosis, thus indicating that these abnormalities are etiology-related in HCC tumors only[24]. In the same way, in the present study, we found transcript dysregulation only in HCC-free cirrhosis and not in peritumoral cirrhosis. We now document that histochemical evaluation of the PLG protein level confirms our results obtained at the transcriptional level. In contrast, for DPF2, dysregulation observed at both transcript and protein levels were in opposite directions, but discrepancies between transcript levels and protein levels have been previously noticed[26,27]. As markers of HCC occurrence are still very scarce[4,28], our observation on transcripts and proteins is of strong interest in early HCC diagnosis. This will need to be further evaluated in HCC-free/cirrhosis-free, fibrotic livers, with selection of marker combinations.

Some up- or down-regulations of transcription factors in HCC have already been documented[29], but the facts that dysregulation of STAT-3 and a related gene network take place in HCC-free cirrhosis, and in an etiology-dependent fashion, are novel findings. The JAK-STAT pathway is critical in the proinflammatory

cytokine-driven inflammatory response provided by hepatocytes[30] and it is tempting to speculate that a weakening of this defense in early cirrhosis may participate in HCC development. However, this mechanism appears unlikely. Indeed, our data were obtained by comparison of alcoholic *vs* HCV cirrhosis samples whose extent of inflammation was similar and still had different STAT-3 regulation. The HCV has a clear effect on the activity of STAT-3, but the meaning of this is controversial. Some studies show inhibition of STAT-3 activity[31] while others show activation of STAT-3[32-34]. Our data are in keeping with documented HCV proteins/STAT-3 interferences and STAT-3 activation in HCV-induced liver disease. STAT-3 directly affects cell proliferation, cell differentiation[35] and angiogenesis[32]. Moreover, STAT-3 and its targets are regulated in some cancers, such as breast and prostate cancer[34]. Thus, the dysregulation of the STAT-3 pathway which follows HCV infection may participate in HCC development at an early stage of hepatocyte dysplasia. In addition, recent reports have highlighted the potential of STAT-3 as a therapeutic target in different neoplasms[36,37].

In conclusion, our data point to major transcription dysregulations in HCC-free cirrhosis. These dysregulations often result from a transient dysregulation, and half are etiology-dependent. Our observations open new avenues for the follow-up of HCC-free cirrhosis because dysregulated transcripts or proteins may be appear like markers for the cirrhosis to HCC transition. In order to complement these results, studies performed at an earlier state before cirrhosis, i.e. on fibrosis samples are now under investigation.

ACKNOWLEDGMENTS

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COMMENTS

Background

Chronic viral hepatitis C (HCV) infection and alcoholism are two important causes of cirrhosis and hepatocellular carcinoma (HCC). Liver transcriptome analysis has resulted in the identification of genes with an aberrant expression according to different pathophysiological states. In the present work, we investigated whether some transcription dysregulations could be found in HCC-free cirrhosis in an etiology-dependent fashion. Furthermore, we searched and found transcription dysregulations that differentiate HCC-free cirrhosis from peritumoral cirrhosis.

Research frontiers

Numerous, genome-wide analyses of abnormal gene expression in HCC as compared to normal, control liver, have resulted in identification of gene sets with altered expression. Few similar studies have been done in HCC-free cirrhosis. In fact, viral etiologies have often been considered whereas abnormal gene expression in alcoholism-dependent HCC has received very little attention. Therefore, the impact of etiology still remains an important issue. We recently reported that a number of genome-wide abnormalities in alcoholism-associated *vs* HCV-associated HCC are etiology-dependent and some of them are of pathological relevance. Remarkably, the abnormal transcript levels that differentiate HCC nodules in an alcoholism-dependent *vs* HCV-dependent HCC can no longer discriminate between the two etiologies when transcripts are measured in the surrounding cirrhosis. Yet, any etiology-dependent abnormalities that could be observed in HCC-free cirrhosis would be of interest.

Applications

These data point to major transient transcription dysregulations in HCC-free cirrhosis. These observations open new avenues for the follow-up of HCC-free cirrhosis because dysregulated transcripts or proteins may be appear like markers for the cirrhosis to HCC transition.

Peer review

The aims of the study were to identify genes that were differentially expressed between HCC-free and HCC-related cirrhosis. The differentially expressed genes were further investigated to see if they were associated with alcoholism and HCV etiologies. The authors suggested that genes that were deregulated in HCC-free cirrhosis might serve as markers for the cirrhosis to HCC transition.

REFERENCES

1 **Pincock S**. Binge drinking on rise in UK and elsewhere. Government report shows increases in alcohol consumption, cirrhosis, and premature deaths. *Lancet* 2003; **362**: 1126-1127
2 **Lee JS**, Thorgeirsson SS. Comparative and integrative functional genomics of HCC. *Oncogene* 2006; **25**: 3801-3809
3 **Thorgeirsson SS**, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002; **31**: 339-346
4 **Bruix J**, Hessheimer AJ, Forner A, Boix L, Vilana R, Llovet JM. New aspects of diagnosis and therapy of hepatocellular carcinoma. *Oncogene* 2006; **25**: 3848-3856
5 **Llovet JM**, Wurmbach E. Gene expression profiles in hepatocellular carcinoma: not yet there. *J Hepatol* 2004; **41**: 336-339
6 **Thorgeirsson SS**, Lee JS, Grisham JW. Molecular prognostication of liver cancer: end of the beginning. *J Hepatol* 2006; **44**: 798-805
7 **Llovet JM**, Chen Y, Wurmbach E, Roayaie S, Fiel MI, Schwartz M, Thung SN, Khitrov G, Zhang W, Villanueva A, Battiston C, Mazzaferro V, Bruix J, Waxman S, Friedman SL. A molecular signature to discriminate dysplastic nodules from early hepatocellular carcinoma in HCV cirrhosis. *Gastroenterology* 2006; **131**: 1758-1767
8 **Laurent-Puig P**, Zucman-Rossi J. Genetics of hepatocellular tumors. *Oncogene* 2006; **25**: 3778-3786
9 **Boyault S**, Rickman DS, de Reyniès A, Balabaud C, Rebouissou S, Jeannot E, Hérault A, Saric J, Belghiti J, Franco D, Bioulac-Sage P, Laurent-Puig P, Zucman-Rossi J. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. *Hepatology* 2007; **45**: 42-52
10 **Laurent-Puig P**, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, Monges G, Thomas G, Bioulac-Sage P, Zucman-Rossi J. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. *Gastroenterology* 2001; **120**: 1763-1773
11 **Villanueva A**, Newell P, Chiang DY, Friedman SL, Llovet JM. Genomics and signaling pathways in hepatocellular carcinoma. *Semin Liver Dis* 2007; **27**: 55-76
12 **Kim JW**, Ye Q, Forgues M, Chen Y, Budhu A, Sime J, Hofseth LJ, Kaul R, Wang XW. Cancer-associated molecular signature in the tissue samples of patients with cirrhosis. *Hepatology* 2004; **39**: 518-527
13 **Edamoto Y**, Hara A, Biernat W, Terracciano L, Cathomas G, Riehle HM, Matsuda M, Fujii H, Scoazec JY, Ohgaki H. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *Int J Cancer* 2003; **106**: 334-341
14 **Shackel NA**, McGuinness PH, Abbott CA, Gorrell MD, McCaughan GW. Novel differential gene expression in human cirrhosis detected by suppression subtractive hybridization. *Hepatology* 2003; **38**: 577-588
15 **Okabe H**, Satoh S, Kato T, Kitahara O, Yanagawa R,

Yamaoka Y, Tsunoda T, Furukawa Y, Nakamura Y. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res* 2001; **61**: 2129-2137

16 **Iizuka N**, Oka M, Yamada-Okabe H, Mori N, Tamesa T, Okada T, Takemoto N, Tangoku A, Hamada K, Nakayama H, Miyamoto T, Uchimura S, Hamamoto Y. Comparison of gene expression profiles between hepatitis B virus- and hepatitis C virus-infected hepatocellular carcinoma by oligonucleotide microarray data on the basis of a supervised learning method. *Cancer Res* 2002; **62**: 3939-3944

17 **Iizuka N**, Oka M, Yamada-Okabe H, Hamada K, Nakayama H, Mori N, Tamesa T, Okada T, Takemoto N, Matoba K, Takashima M, Sakamoto K, Tangoku A, Miyamoto T, Uchimura S, Hamamoto Y. Molecular signature in three types of hepatocellular carcinoma with different viral origin by oligonucleotide microarray. *Int J Oncol* 2004; **24**: 565-574

18 **Iizuka N**, Oka M, Yamada-Okabe H, Mori N, Tamesa T, Okada T, Takemoto N, Hashimoto K, Tangoku A, Hamada K, Nakayama H, Miyamoto T, Uchimura S, Hamamoto Y. Differential gene expression in distinct virologic types of hepatocellular carcinoma: association with liver cirrhosis. *Oncogene* 2003; **22**: 3007-3014

19 **Kurokawa Y**, Matoba R, Takemasa I, Nakamori S, Tsujie M, Nagano H, Dono K, Umeshita K, Sakon M, Ueno N, Kita H, Oba S, Ishii S, Kato K, Monden M. Molecular features of non-B, non-C hepatocellular carcinoma: a PCR-array gene expression profiling study. *J Hepatol* 2003; **39**: 1004-1012

20 **Yoon SY**, Kim JM, Oh JH, Jeon YJ, Lee DS, Kim JH, Choi JY, Ahn BM, Kim S, Yoo HS, Kim YS, Kim NS. Gene expression profiling of human HBV- and/or HCV-associated hepatocellular carcinoma cells using expressed sequence tags. *Int J Oncol* 2006; **29**: 315-327

21 **Coulouarn C**, Gomez-Quiroz LE, Lee JS, Kaposi-Novak P, Conner EA, Goldina TA, Onishchenko GE, Factor VM, Thorgeirsson SS. Oncogene-specific gene expression signatures at preneoplastic stage in mice define distinct mechanisms of hepatocarcinogenesis. *Hepatology* 2006; **44**: 1003-1011

22 **Geiss GK**, Carter VS, He Y, Kwieciszewski BK, Holzman T, Korth MJ, Lazaro CA, Fausto N, Bumgarner RE, Katze MG. Gene expression profiling of the cellular transcriptional network regulated by alpha/beta interferon and its partial attenuation by the hepatitis C virus nonstructural 5A protein. *J Virol* 2003; **77**: 6367-6375

23 **Osna NA**, White RL, Todero S, McVicker BL, Thiele GM, Clemens DL, Tuma DJ, Donohue TM Jr. Ethanol-induced oxidative stress suppresses generation of peptides for antigen presentation by hepatoma cells. *Hepatology* 2007; **45**: 53-61

24 **Derambure C**, Coulouarn C, Caillot F, Daveau R, Hiron M, Scotte M, Francois A, Duclos C, Goria O, Gueudin M, Cavard C, Terris B, Daveau M, Salier JP. Genome-wide differences in hepatitis C- vs alcoholism-associated hepatocellular carcinoma. *World J Gastroenterol* 2008; **14**: 1749-1758

25 **Coulouarn C**, Lefebvre G, Derambure C, Lequerre T, Scotte M, Francois A, Cellier D, Daveau M, Salier JP. Altered gene expression in acute systemic inflammation detected by complete coverage of the human liver transcriptome. *Hepatology* 2004; **39**: 353-364

26 **Greenbaum D**, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol* 2003; **4**: 117

27 **Unwin RD**, Whetton AD. Systematic proteome and transcriptome analysis of stem cell populations. *Cell Cycle* 2006; **5**: 1587-1591

28 **Di Tommaso L**, Franchi G, Park YN, Fiamengo B, Destro A, Morenghi E, Montorsi M, Torzilli G, Tommasini M, Terracciano L, Tornillo L, Vecchione R, Roncalli M. Diagnostic value of HSP70, glypican 3, and glutamine synthetase in hepatocellular nodules in cirrhosis. *Hepatology* 2007; **45**: 725-734

29 **Xu L**, Hui L, Wang S, Gong J, Jin Y, Wang Y, Ji Y, Wu X, Han Z, Hu G. Expression profiling suggested a regulatory role of liver-enriched transcription factors in human hepatocellular carcinoma. *Cancer Res* 2001; **61**: 3176-3181

30 **Ruminy P**, Gangneux C, Claeyssens S, Scotte M, Daveau M, Salier JP. Gene transcription in hepatocytes during the acute phase of a systemic inflammation: from transcription factors to target genes. *Inflamm Res* 2001; **50**: 383-390

31 **Stärkel P**, Saeger CD, Leclercq I, Horsmans Y. Role of signal transducer and activator of transcription 3 in liver fibrosis progression in chronic hepatitis C-infected patients. *Lab Invest* 2007; **87**: 173-181

32 **Nasimuzzaman M**, Waris G, Mikolon D, Stupack DG, Siddiqui A. Hepatitis C virus stabilizes hypoxia-inducible factor 1alpha and stimulates the synthesis of vascular endothelial growth factor. *J Virol* 2007; **81**: 10249-10257

33 **Waris G**, Turkson J, Hassanein T, Siddiqui A. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. *J Virol* 2005; **79**: 1569-1580

34 **Alvarez JV**, Febbo PG, Ramaswamy S, Loda M, Richardson A, Frank DA. Identification of a genetic signature of activated signal transducer and activator of transcription 3 in human tumors. *Cancer Res* 2005; **65**: 5054-5062

35 **Subramaniam PS**, Torres BA, Johnson HM. So many ligands, so few transcription factors: a new paradigm for signaling through the STAT transcription factors. *Cytokine* 2001; **15**: 175-187

36 **Brantley EC**, Benveniste EN. Signal transducer and activator of transcription-3: a molecular hub for signaling pathways in gliomas. *Mol Cancer Res* 2008; **6**: 675-684

37 **Costantino L**, Barlocco D. STAT 3 as a target for cancer drug discovery. *Curr Med Chem* 2008; **15**: 834-843

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ORIGINAL ARTICLES

Effects of ciglitazone and troglitazone on the proliferation of human stomach cancer cells

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Abstract

AIM: To determine the cytological and molecular effects of peroxisome proliferation-activated receptor (PPAR)-γ and PPAR-γ agonists on stomach cancer cells.

METHODS: To determine the proliferation-suppressive effects of troglitazone and ciglitazone, SNU-216 and SNU-668 stomach cancer cells were plated in media containing 40 μmol/L troglitazone and ciglitazone at a density of 1×10^4 cells/well. After 3, 5 and 7 d, the cells were counted with a hemocytometer. To assess the appearance of PPAR-γ, a reverse-transcription polymerase chain reaction analysis was performed. On day 7, Western blotting was used to determine the effects of troglitazone and ciglitazone on the expression of *p21* and phosphorylated-ERK (*pERK*) genes. Flow cytometry analysis was used to determine which portion of the cell cycle was delayed when troglitazone was used to suppress cell proliferation. In order to clarify the mechanism underlying the activity of troglitazone, microarray analysis was conducted.

RESULTS: PPAR-γ was manifested in both SNU-216 and SNU-668 cells. Ciglitazone and troglitazone suppressed cell growth, and troglitazone was a stronger suppressor of stomach cancer cells than ciglitazone, an inducer of cell cycle arrest in the G1 phase. SNU-668 cells were also determined to be more sensitive to ciglitazone and troglitazone than SNU-216 cells. When troglitazone and ciglitazone were administered to stomach cancer cells, levels of p21 expression were increased, but ERK phosphorylation levels were reduced. When GW9662, an antagonist of PPAR-γ, was applied in conjunction with ciglitazone and troglitazone, the cell growth suppression effect was unaffected. The gene transcription program revealed a variety of alterations as the consequence of troglitazone treatment, and multiple troglitazone-associated pathways were detected. The genes whose expression was increased by troglitazone treatment were associated with cell development, differentiation, signal transmission between cells, and cell adhesion, and were also associated with reductions in cell proliferation, the cell cycle, nuclear metabolism, and phosphorylation.

CONCLUSION: Troglitazone and ciglitazone suppress the proliferation of stomach cancer cells *via* a PPAR-γ-independent pathway.

Key words: Peroxisome proliferating-activated receptor-γ; Ciglitazone; Troglitazone; Stomach cancer cells

Peer reviewer: Sharon DeMorrow, Assistant Professor, Division of Research and Education, Scott and White Hospital and The Texas A&M University System, Health Science Center College of Medicine, Temple, Texas 76504, United States

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INTRODUCTION

The peroxisome proliferator-activated receptors (PPARs) are members of the family of nuclear receptors[1], which themselves constitute a group in the steroid/thyroid hormone/retinoid receptor superfamily. Their action mechanisms induce the formation of a heterodimer after PPAR unites with its ligand and retinoid X receptor (RXR) in the nucleus, which subsequently activates the manifestation of genetic DNA by working on the transcription factors of promoter sites. Thus far, three

subtypes, α, β/δ, and γ, have been identified, and an increasing quantity of research into PPAR-γ has been conducted since the initial detection of the composed ligand[2]. According to the research conducted over the last 10 years, PPAR-γ has been associated with novel functions in cell division and differentiation, functions crucial to inflammation, tissue resuscitation, vascular biology, cancer formation, and apoptosis[3]. As a result of these functions, the PPARs have been implicated as a treatment factor for diabetes mellitus, metabolic syndrome, atherosclerosis, and certain types of cancer[2,4]. PPAR-γ has been detected in a broad variety of cancers, including colon, breast, lung and prostate cancer. Ligands of PPAR-γ have been demonstrated to suppress the propagation of these cancers *in vitro*[5-8]. The results of this study suggest that many human malignant cancers may eventually be cured using PPAR-γ ligands. One well-known category of ligands is the thiazolidinediones (TZDs), which includes rosiglitazone, troglitazone, ciglitazone and 15-deoxy-prostaglandin-J2 (15d-PGJ2)[9].

Stomach cancer is one of the most common cancers worldwide. The condition is not so common in America and Europe, but is relatively common in Asia, and specifically in South Korea. A great many drugs already exist for treatment of stomach cancer, and these have already brought great improvements in survival rates and quality of life. However, there is currently no standard protocol by which personal sensitivity or resistance to treatment can be predicted. Lu *et al*[10] has previously reported that troglitazone suppresses stomach cancer *via* the activation of PPAR-γ, and in another study, it has been reported that stomach cancer is suppressed by PPAR-γ-ligand-mediated apoptosis[11].

The PPAR-γ ligand has two different pathways, one of which is PPAR-γ-dependent, and one PPAR-γ-independent[10,12-18]. The relationship between the independent pathway and stomach cancer has been confirmed, for example, by the finding that the 15d-PGJ2-induced suppression of colon cancer cells can be achieved *via* the manifestation of Kruppel-like factor 4 (KLF4)[16].

The principal objective of the present study was to determine the mechanism underlying the activity of PPAR-γ. After we confirmed the activation of PPAR-γ in two types of stomach cancer cells and administration of ciglitazone and troglitazone, both of which induce PPAR-γ activation, we were able to make an observation about cell proliferation, confirm the effects of PPAR-γ suppressors, and clarify any genetic alterations *via* the use of cDNA microarrays.

MATERIALS AND METHODS

Materials

We utilized troglitazone, ciglitazone, GW9662, propidium iodide, and dimethyl sulfoxide (DMSO) obtained from Sigma Co. (St. Louis, MO, USA), RPMI 1640, fetal bovine serum (FBS), 0.05% trypsin/0.02% EDTA, penicillin/streptomycin from Invitrogen Co. (Grand Island, NY, USA) and total-ERK, phosphorylated-ERK, and p21 antibody from Cell Signaling Technology Co. (Beverly, MA, USA). Troglitazone and ciglitazone solution was added at a concentration of 40 μmol/L per well. When adding the materials, we utilized DMSO solution and ensured identical conditions and DMSO concentration between the control and experimental groups.

Cultivation of cell strains

The SNU-216 and SNU-668 stomach cancer cell strains were obtained from the Korean Cell Bank (Seoul National University Hospital, Cancer Institute, Seoul, Korea) and were used as cultured. Cell culture was carried out at 37℃ in an atmosphere of 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Measurement of vegetative function

In order to determine the proliferation-suppressive effects of troglitazone and ciglitazone, after washing a growth phase cell strain, we separated cells with 0.05% trypsin/0.02% EDTA. These cells were mixed thoroughly and cultured for 24 h in six-well plates at a concentration of 1×10^4 cells/well. We verified the attachment of the cells to the plates, and then added 40 μmol/L troglitazone and ciglitazone to each 10% FBS medium. After 3, 5 and 7 d, we separated the proliferated cells with 0.05% trypsin/0.02% EDTA. These cells were counted with a hemocytometer and compared with the control group to assess the suppressive effects on cell growth.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

After washing the cultured cells with Hank's Buffered (or Balanced) Salt Solution (HBSS), we briefly mixed them with TRI Reagent (Sigma) and maintained them for 15 min at 4℃. We then mixed them one additional time with 200 μL chloroform and maintained them for an additional 5 min at 4℃. We then subjected the samples to centrifugation at a rate of 12000 rpm at 4℃, transferred the upper layer to a new tube and added an equal volume of isopropanol. This tube was maintained for 5 min at 4℃, and then centrifuged. The samples were dried and dissolved in diethylpyrocarbonate (DEPC)/distilled water, after washing the centrifuged pellets with DEPC/70% ethanol. The RNA was quantitated after determining the optical density at 260 nm, after which the reverse transcription reaction was conducted. Distilled water was added and settled with buffer (Promega, MO, USA) with 20 μmol/L dNTP, 0.25 μg oligo (dT) 15 primer, 5 U Avian Myoblastosis Virus (Promega), reverse transcriptase (Promega) and 2 μg RNA and DEPC. We established the total quantity at 20 μL and the reaction was performed for 60 min at 42℃.

We used an AccuPower PCR Premix (BIONEER, Seoul, Korea) kit. After the reverse transcription reaction was finished, we added 1 μL RT product and 10 pmol

sense and antisense primers into the tube provided in the kit. We established the total volume at 20 μL with distilled water and initiated the PCR.

Western blot analysis

After washing the cultured cells in HBSS, the cells were lysed in lysis buffer and then placed on ice for 30 min. The protein was extracted by centrifuging this solution. We then added 4 × loading buffer to the protein standard marker and to each protein (5 μg/μL), and then denatured them for 7 min at 95℃. Electrophoresis was conducted for 2 h at 100 V on 4% and 10% SDS-polyacrylamide gels. After electrophoresis, we placed the gel in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol) for 15 min, and then transferred it to nitrocellulose (NC) membrane for 1 h at 20 V. In order to prevent non-specific antibody binding, we placed the NC membrane in blocking solution, 5% non-fat milk dissolved in Tris-buffered saline (TBS), and incubated the reactions with slight shaking. We then placed them into prepared primary antibody diluted 1/1000 with TBS/Tween buffer (TBS buffer with 0.02% Tween 20) including 5% non-fat milk, and then added it to the NC membranes overnight at 4℃. The samples were washed three times for 10 min each. We diluted the secondary antibody, anti-rabbit IgG in 5% non-fat milk to dilute it 1/1000, immersed the NC membrane in this solution with shaking for 90 min, and then washed the samples three times with TBS/Tween buffer for 10 min each. We subjected the NC membranes to enhance chemiluminescence (Amersham, Buckinghamshire, UK) for 5 min, exposed them to light for 10 min in a darkroom, and then detected the signals on the films. After visual certification of the luminosity and intensity of the bands, we quantified the intensity of the bands using a Gel Image Analysis System (UVItec, Cambridge, UK).

Cell cycle analysis *via* flow cytometry

We conducted flow cytometry analysis to determine which portion of the cell cycle was delayed when we used troglitazone to suppress the proliferation of SNU-216 and SNU-668 cells. Cultured SNU-216 and SNU-668 cell strains were washed in HBSS. We then added 40 μmol/L troglitazone, and fixed the cultures for 30 min with 70% ethanol at 4℃. After fixation, we degraded the RNA with RNase A (Sigma) and dyed the DNA with propidium iodide in order to prepare intercalated DNA. The cell cycles were compared and analyzed using a Becton-Dickinson FACStar Flow Cytometer and Becton-Dickinson Cell Fit Software (Becton-Dickinson, Erenbode, Belgium).

Illumina microarray

Microarray analysis was conducted using an Illumina BeadStation 500 X manual system, obtained from Microgen Co. (Seoul, Korea). We prepared the biotinylated cRNA with an Illumina Amplification Kit (Ambion, CA, USA), and refined it with an RNeasy Kit (Qiagen, CA, USA). Hybridization was conducted with a Sentrix HumanRef-8 Expression BeadChip system (Illumina, CA USA), which included approximately 24 000 probes, and conducted lavation in accordance with the manufactures instructions, followed by scanning with a confocal laser scanner (Nikon Precision Korea, Yong-In, Korea). We then conducted statistical analysis using Avadis Prophetic software, version 3.3 (Strand Genomics, Bangalore, India).

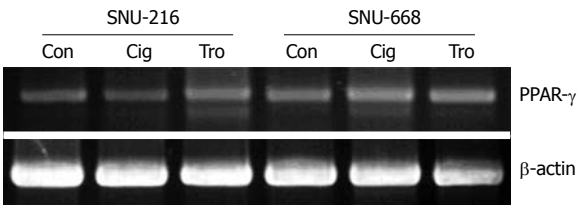

Figure 1 PPAR-γ expression was confirmed by RT-PCR in human gastric cancer cell lines (SNU-216 and SNU-668) treated with troglitazone (Tro) or ciglitazone (Cig) and the β-actin control (Con) is shown in the bottom panel.

Statistical analysis

One-way analysis of variance and Fisher's LSD test were used to compare statistical differences between each group, and a P value < 0.05 was considered significant for all statistical analyses.

RESULTS

The manifestation of PPAR in SNU-216 and SNU-668 stomach cancer cells

As a result of the manifestation of PPAR-γ in stomach cancer cells SNU-216 and SNU-668 using RT-PCR, both cells were confirmed to be positive, and no significant differences were detected (Figure 1). Additionally, the difference in the degree of manifestations was not significantly different when troglitazone and ciglitazone were applied at 40 μmol/L for 7 d, and the manifestations of PPAR-γ were assessed *via* RT-PCR (Figure 1).

Changes in cell morphology by settlement of troglitazone and ciglitazone

We observed cell morphology after exposing SNU-216 and SNU-668 cells to 40 μmol/L troglitazone and ciglitazone for 7 d. The SNU-216 cells showed no significant morphological changes after treatment with the two compounds. On the contrary, the SNU-668 cells demonstrated morphological changes after 2 d troglitazone, but not ciglitazone, treatment; the cells were lengthened at both ends, and assumed a spindle-type morphology (Figure 2).

Troglitazone- and ciglitazone-induced inhibition of SNU-216 and SNU-668 cell proliferation

In order to assess the suppressive effects of troglitazone and ciglitazone on the proliferation of SNU-216 and

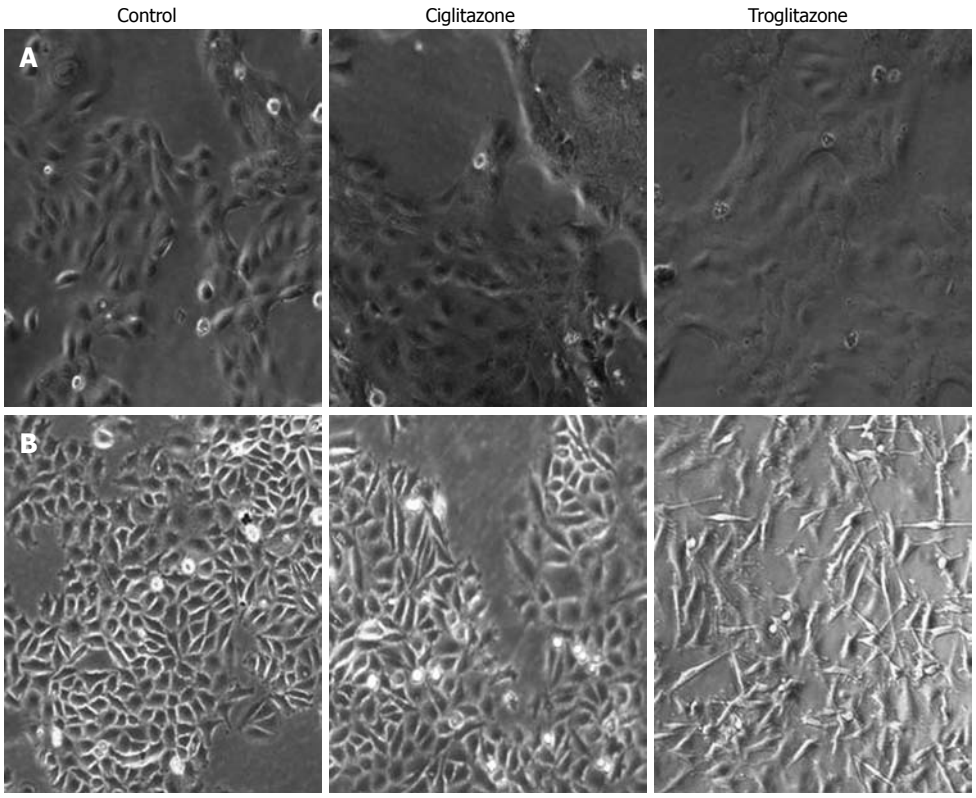

Figure 2 Change in cell morphology after treatment with troglitazone or ciglitazone. There were more significant differences in SNU-668 (B) than SNU-216 (A) cells, as shown by inverted microscopy (original magnification, x 100).

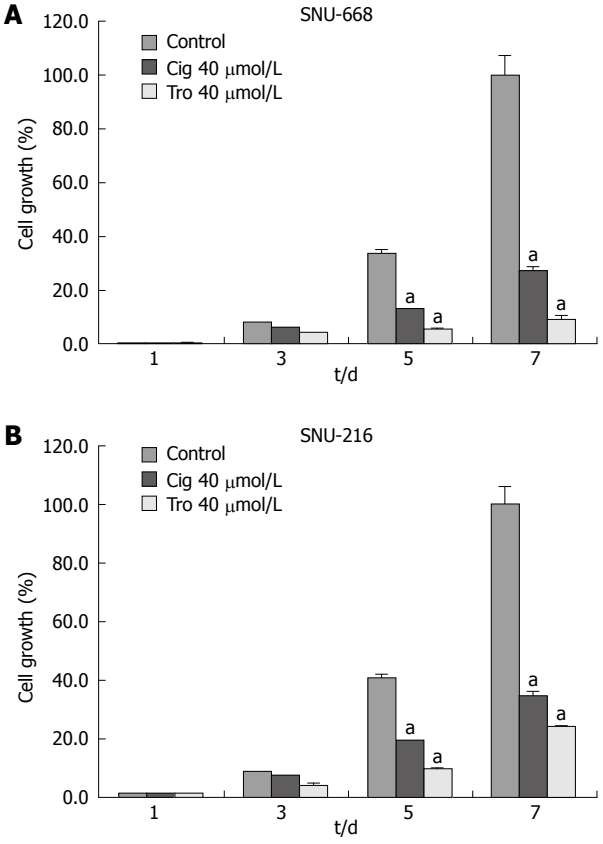

Figure 3 Growth inhibition by troglitazone or ciglitazone in human gastric cancer cell lines. There was a more significant increase of suppressive effect in SNU-668 (A) than SNU-216 (B) cells compared with the control group. $^{a}P < 0.05$ *vs* control group.

SNU-668 cells, we cultured the cells for 24 h on six-well plates at 1×10^4 cells/well. We added troglitazone and ciglitazone to the medium, and cultured the cells again. We counted the number of cells at 3, 5 and 7 d after culturing. The growth rate of the SNU-216 cells was reduced at 3, 5 and 7 d after troglitazone treatment, and the cell count percentages were 45%, 24% and 24%, as compared with the control group. The growth rate of the SNU-668 cells was reduced on the same days by troglitazone treatment, by 49%, 15% and 9%. However, with ciglitazone treatment, the growth rate of the SNU-688 strain was reduced less profoundly, and the percentages of the cell count were 77%, 38% and 27%. As a result, troglitazone appeared to exert a more profound suppressive effect than did ciglitazone (Figure 3). Ciglitazone and troglitazone treatment did not significantly affect SNU-216 and SNU-668 cell death (Figure 4).

Cell cycle analysis using flow cytometry

We assessed the cell cycles of the two groups. One group was SNU-216 and SNU-668 cells cultured in media to which 40 μmol/L troglitazone and ciglitazone was added, and the other group was a control group that was cultured without any drug treatment. In the SNU-216 cells, each of the G0/G1 phases were 77%, 78% and 79%, the S phases were all 11%, and the G2/M phases were 12%, 11% and 10%. However, in the SNU-668 cells, the G0/G1 phases of each group were 73%, 76% and 86%, the S phases were 11%, 11% and 8%, and the G2 phases were 16%, 12% and 6% (Figure 5).

Effect of troglitazone and ciglitazone on expression of p21 and pERK genes in SNU-216 and SNU-668 cells

We cultured SNU-216 and SNU-668 cells at a

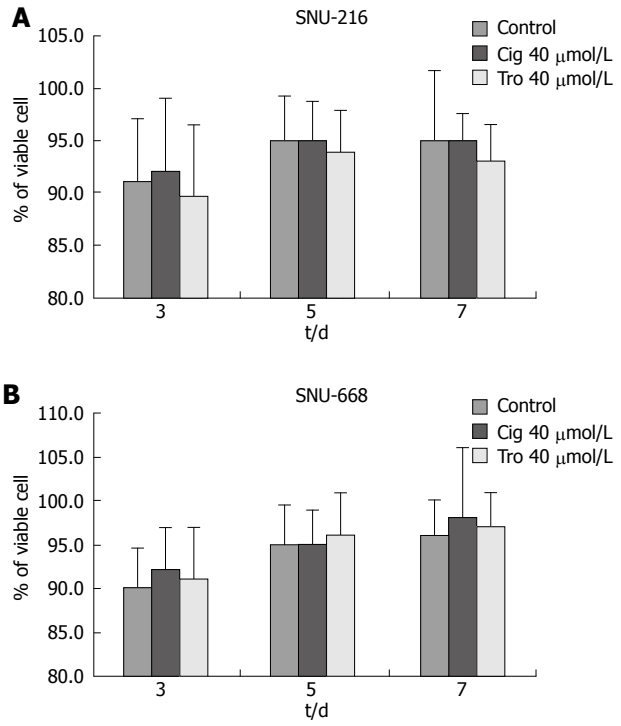

Figure 4 Cell viability measured by hemocytometry. Viability of the troglitazone- or ciglitazone-treated cells was decreased more than that of the control group, with no significant difference between the two cell lines.

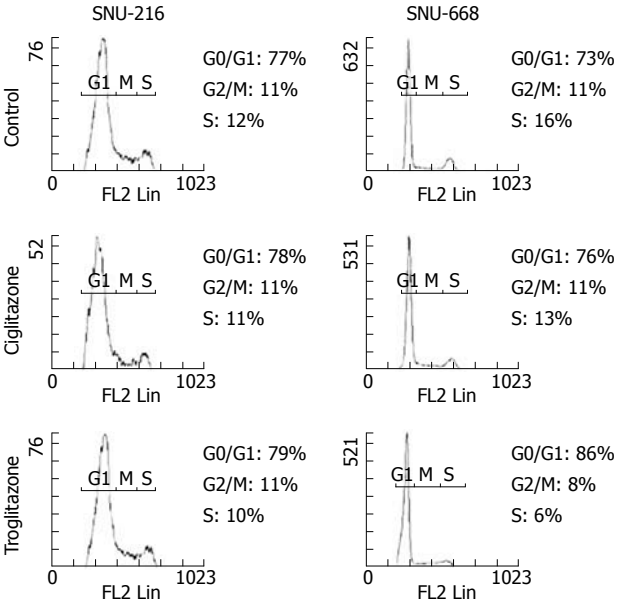

Figure 5 Effects of troglitazone and ciglitazone on cell cycle distribution measured by flow cytometry. It shows meaningful arrest during G2/M and S phase in SNU-668 treated with triglitazone.

concentration of 1×10^4 cells/well on six-well plates for 24 h, and added troglitazone and ciglitazone at levels of up to 40 μmol/L for 7 d. On day 7, we conducted Western blotting after extracting the proteins with from the cultured cells for 7 d, and assessed the density of the bands *via* image analysis. As a result of the expression of p21, we noted no significant interval changes before and after drug treatment in SNU-216 cells. In SNU-668 cells, we noted an increase of approximately 2.8-fold

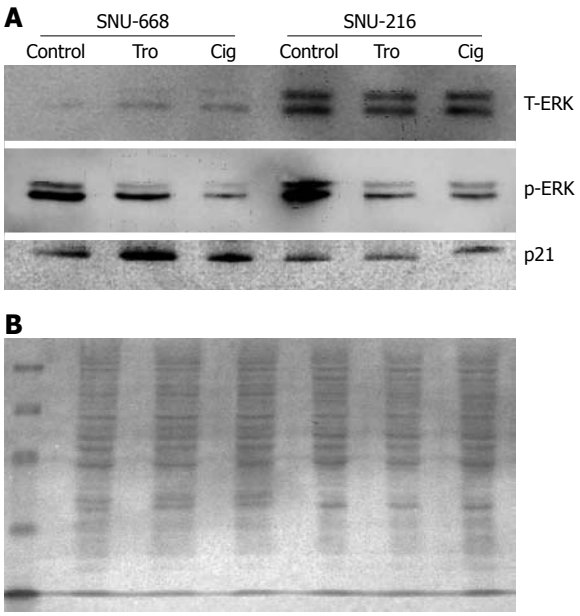

Figure 6 Western blot analysis for the expression of total-ERK, p-ERK and p21. A: There was a significant decrease in phosphorylation of ERK and increased expression of p21 in SNU-668 cells with ciglitazone or troglitazone treatment. B: Ponceau S protein staining of the membrane of SNU-216 and SNU-668 cells to ensure equal loading of protein in the sample.

with troglitazone, and a 1.6-fold increase with ciglitazone. Phosphorylation of ERK was significantly reduced by ciglitazone and troglitazone (Figure 6).

Influence of PPAR-γ antagonists on the suppressive effects of troglitazone and ciglitazone on cell growth

In order to evaluate the association between the suppressive effects of cell growth with identical levels of troglitazone, ciglitazone and PPAR-γ activation, we confirmed the effects of GW9662[19], which has been previously identified as a selective PPAR-γ suppressor. After culturing the solution at 1×10^4 cells/well in six-well plates, we added antagonist 5 μmol/L GW9662, 5 μmol/L GW9662 + 40 μmol/L ciglitazone, 5 μmol/L GW9662 + 40 μmol/L troglitazone to each medium, and counted the cells 3, 5, and 7 d later. The suppressive effects of 40 μmol/L troglitazone and ciglitazone was not influenced by treatment with 5 μmol/L GW9662 (Figure 7).

Changes in gene expression

To elucidate the mechanism underlying the activity of troglitazone, we evaluated the troglitazone-induced changes in gene expression, *via* the microarray technique. We verified these changes, and found that expression of 388 genes was increased by more than two-fold, and 466 were reduced by more than two-fold. According to the analysis of genetic functions with genetic manifestation by PANTHER (Protein Analysis Through Evolutionary Relationsips) Classification System (SRI international, CA, USA), cell cycle, DNA metabolism, somatic cell division, replication, and DNA repair were suppressed to a significant degree. However, signal transduction and homeostasis were increased (Table 1). In an effort to determine the categories of biological processes affected

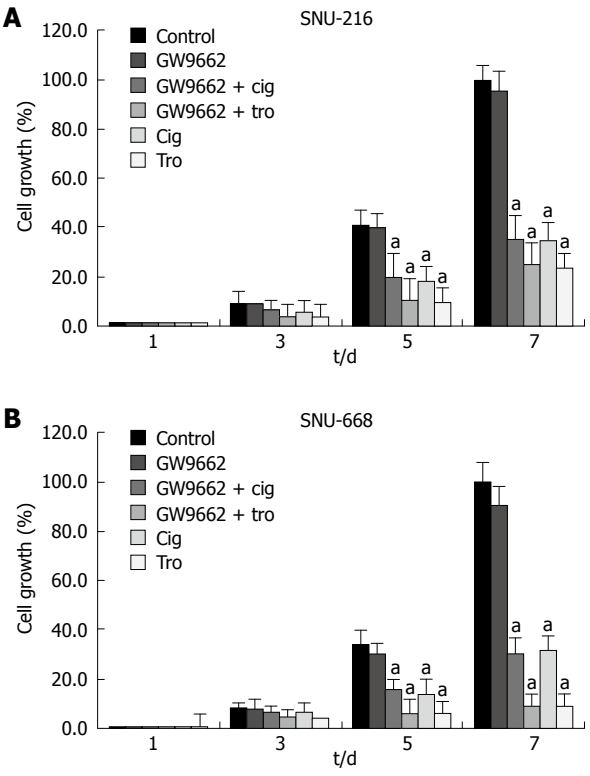

Figure 7 Effect of GW9662 on the inhibition of cell proliferation. GW9662, a selective PPAR-γ suppressor, had no effect on ciglitazone- or troglitazone-induced inhibition of cell proliferation. $^{a}P < 0.05$ *vs* control group.

by troglitazone treatment, we conducted functional annotation analyses using DAVID bioinformatic tools[20], with the genes whose expression was altered more than two-fold. The results of this analysis reveal a distinct distribution of biological processes between these genes (Tables 2 and 3). The expression of genes associated with signal transmission for cell communication, growth, differentiation, and cell adhesion was increased. Additionally, expression of genes associated with cell proliferation, the cell cycle, nuclear metabolism and phosphorylation was also reduced. The functional differences between the increased and reduced genes were confirmed by the changes in the KEGG pathway, as detected by DAVID analysis. The pathway underlying the reductions in the levels of these genes was associated with the cell cycle, DNA polymerase, and purine and pyrimidine metabolism, the effects of which on the genes with increased expression was not confirmed (Table 4).

DISCUSSION

PPAR-γ is manifested in a variety of tissues and cancer cells[21], and the ligands that activate PPAR-γ are currently being studied as a possible novel therapeutic modality[19,22-26]. The PPAR-γ ligand generally reduces the survival rate of cancer cells *via* differentiation, apoptotic induction, and changes in genes or proteins associated with entrance into the G1/S phase[15,27]. Some reports have suggested that stomach cancer cells manifest PPAR-γ and are suppressed by PPAR-γ ligands[11]. Recently, another study has demonstrated that troglitazone, a PPAR-γ ligand, may prove useful in preventive medicine, and this effect appears to occur in a PPAR-γ-dependent manner[10].

Table 1 Alteration of biological processes in SNU-668 cells treated with troglitazone as compared with controls

Biological process	Number	Over/under	*P* value
Cell cycle	116	-	3.510E-13
DNA metabolism	57	-	1.150E-09
Mitosis	38	-	1.060E-07
DNA replication	38	-	7.350E-06
DNA repair	23	-	1.110E-03
Cell surface receptor mediated signal transduction	52	+	1.850E-03
Cytokinesis	10	-	3.280E-03
Chromosome segregation	14	-	3.300E-03
Homeostasis	14	+	9.100E-03
Signal transduction	133	+	1.230E-02
Transport	41	+	1.740E-02
Nucleoside, nucleotide and nucleic acid metabolism	165	-	1.800E-02

The results of our study show that ciglitazone and troglitazone suppress the proliferation of stomach cancer cells *via* G1 phase arrest. The arrest of G1 phase has been reported in colon cancer cells as the result of PPAR-γ activation[12]. In the present study, each of the stomach cancer cell types that showed p53 mutations was suppressed more strongly by troglitazone than by ciglitazone, and this effect was detected more prominently in the SNU-668 cells than in the SNU-216 cells. The SNU-668 cells demonstrated p53 and ras mutations, and it will be necessary in future studies to clarify the relationship between these results and the medical mechanisms underlying them.

Few investigations have thus far addressed the induction mechanism by which stomach cancer cell proliferation is suppressed by troglitazone. As the results of some studies have suggested that PPAR agonists induce apoptosis in cancer cells, the observed cell-proliferation-suppressive effects may derive from a reduction in cell consistency. This indicates that the reduction in cells as the result of apoptosis is not very relevant to the aforementioned cell-proliferation-suppressive effects.

Jung *et al*[28] have evaluated the reaction of the PPAR-γ agonist pathway on PPAR-γ. For example, troglitazone suppresses the proliferation of colon cancer cells, induces apoptosis, and induces the growth response-1 gene in early phase. These processes are activated downstream of the suppression in a one-by-one fashion, *via* a PPAR-γ-independent pathway[29]. Chintharlapalli *et al*[17,18] have reported that an inductive chemical compound associated with CDDO induces both PPAR-γ-dependent caveolin manifestation and a PPAR-γ-independent induction of apoptosis. In our study, we determined that this mechanism occurs *via* an independent pathway, as suppression of the PPAR-γ agonist was not suppressed by PPAR-γ antagonists.

We utilized different concentrations of troglitazone to induce growth suppression, as has been done for

Table 2 Biological process of up-regulated genes by triglitazone in SNU-668 cells

Term	Count	*P* value	Genes
Negative regulation of cellular process	29	2.26E-04	ANGPTL4, ZFHX1B, MXD4, CXCL1, GPNMB, CDKN1A, IRF1, SESN2, JAZF1, FTH1, IL6, SVIL, JARID1B, MN1, DDIT3, PPP1R15A, IER3, ARHGEF2, TNFAIP3, RHOB, PRDM1, QSCN6, BNIP3L, MAP4, NRG1, EREG, FAIM3, IL8, FST
Response to chemical stimulus	20	1.91E-04	CCL20, PLD1, STC2, APOE, DEFB1, PLAU, CXCL1, SEMA3C, SRXN1, CCL5, SOD2, HSPA6, STC1, CXCL2, NDRG1, DNAJB2, IL8, MVP, ECGF1, ARNT2
Chemotaxis	8	0.009882707	CCL20, PLD1, CXCL2, CXCL1, PLAU, IL8, ECGF1, CCL5
Negative regulation of cell proliferation	9	0.009171778	FTH1, IL6, MXD4, EREG, CXCL1, IL8, GPNMB, CDKN1A, QSCN6
Programmed cell death	22	0.002544517	SQSTM1, TNFRSF21, PPP1R15A, APOE, ANGPTL4, IER3, TNFAIP3, RHOB, TNFRSF10A, ROCK1, HIPK2, CDKN1A, BNIP3L, C10orf97, IL6, RRAGC, APP, PHLDA2, RIPK2, FAIM3, IL24, TRIB3
Development	62	9.62E-06	SERPINE2, CXCL1, PHGDH, EPC1, MSX1, IL6, SLC3A2, COL7A1, IFRD1, NAV1, LIF, IER3, ARHGEF2, RHOB, DHRS9, PKD1, VAT1, SEMA3C, QSCN6, ANPEP, LTBP4, EDG3, SOX9, SQSTM1, PTGS2, IGF2BP3, FBN1, DNER, IGFBP5, CTGF, ANGPTL4, APOE, ZFHX1B, CSPG2, IL11, KLF6, RRAGC, S100P, NDRG1, SVIL, PNMA1, DACT1, CYP1B1, WNT5A, MAFB, PDLIM7, LAMA4, CRMP1, PAPPA, CAMTA1, RUNX1, KRTHA4, EREG, GPR56, NRG1, FST, IL8, DCN, ECGF1, ARNT2, NRP1, ITGA2
Organelle organization and biogenesis	28	0.005845300	WASPIP, APOE, HIST1H1C, CXCL1, EPC1, SVIL, MICAL1, H2BFS, HIST2H2AA, FMNL2, TINF2, PLEC1, TUBA1, HIST2H2BE, ARHGEF2, RHOF, ROCK1, TMOD1, HIST1H2BK, RHOB, SLC22A4, FSCN1, HIST1H2BD, ATG4A, KIF1A, MAP4, ECGF1, KLHL5
Inflammatory response	12	0.002509065	CCL20, PTGS2, CXCL2, PLA2G4C, CEBPB, EDG3, CXCL1, RIPK2, IL8, TNFAIP6, IRAK2, CCL5
Organ development	21	0.003134980	FBN1, ANGPTL4, PDLIM7, RHOB, PHGDH, IL11, ANPEP, MSX1, IL6, KLF6, SVIL, EREG, GPR56, SOX9, DCN, IL8, ECGF1, CYP1B1, NRP1, ITGA2, IFRD1
Transcription from RNA polymerase Ⅱ promoter	17	0.024568720	SQSTM1, TRAK1, TSC22D1, DDIT3, MAFF, MXD4, IRF1, PRDM1, ATBF1, JAZF1, SOD2, FOXF1, NFIL3, RUNX1, STAT5A, CEBPB, SOX9
Cellular morphogenesis	12	0.016732354	KLF6, RRAGC, CTGF, IGFBP5, APOE, LTBP4, ARHGEF2, SLC3A2, VAT1, NRP1, QSCN6, EPC1
Blood vessel morphogenesis	7	0.002345097	ANPEP, ANGPTL4, EREG, RHOB, IL8, ECGF1, NRP1
Response to wounding	20	2.23E-04	CCL20, PTGS2, CTGF, PLA2G4C, PLAU, CXCL1, TNFAIP6, CCL5, SOD2, IL11, CXCL2, CEBPB, EREG, EDG3, RIPK2, FAIM3, IL8, SERPINE1, IRAK2, ITGA2
Apoptosis	22	0.002437855	SQSTM1, TNFRSF21, PPP1R15A, APOE, ANGPTL4, IER3, TNFAIP3, RHOB, TNFRSF10A, ROCK1, HIPK2, CDKN1A, BNIP3L, C10orf97, IL6, RRAGC, APP, PHLDA2, RIPK2, FAIM3, IL24, TRIB3
Cell-cell signaling	24	2.63E-04	CCL20, PBEF1, STC2, APOE, STX1A, LIF, GDF15, MAOA, TNFAIP6, GRB10, CCL5, IL11, STC1, GABARAPL1, IL6, LTBP4, EREG, GPR56, EFNA1, FST, IL8, ECGF1, NRP1, WNT5A
Response to stress	35	0.003636369	SQSTM1, CCL20, PTGS2, ERRFI1, PLA2G4C, CTGF, ANGPTL4, APOE, CXCL1, CD83, SRXN1, TNFAIP6, CCL5, IL11, SOD2, HSPA6, IL6, CD55, CEBPB, DNAJB2, SERPINE1, CFB, PPP1R15A, DDIT3, DEFB1, PLAU, CXCL2, EREG, EDG3, RIPK2, FAIM3, IL8, ARNT2, IRAK2, ITGA2
Vasculature development	7	0.002345097	ANPEP, ANGPTL4, EREG, RHOB, IL8, ECGF1, NRP1
Regulation of cell proliferation	16	6.45E-04	PBEF1, LIF, MXD4, ARHGEF2, CXCL1, GPNMB, CDKN1A, QSCN6, IL11, IRS2, FTH1, IL6, EREG, EDG3, IL8, NRP1
Cell organization and biogenesis	47	0.001133913	SQSTM1, TRAK1, WASPIP, STX3A, IGFBP5, CTGF, APOE, HIST1H1C, CXCL1, DNAJC12, EPC1, GABARAPL1, KLF6, RRAGC, SVIL, LARP6, MICAL1, HIST2H2AA, H2BFS, SLC3A2, FMNL2, TINF2, PLEC1, STX1A, TUBA1, DUSP16, HIST2H2BE, STX11, RHOF, ARHGEF2, RHOB, HIST1H2BK, TMOD1, ROCK1, SLC22A4, VAT1, FSCN1, QSCN6, BET1, HIST1H2BD, ATG4A, KIF1A, LTBP4, MAP4, ECGF1, KLHL5, NRP1
Cell differentiation	21	0.001730483	SQSTM1, PTGS2, APOE, ANGPTL4, PDLIM7, SERPINE2, RHOB, DHRS9, PAPPA, IL11, ANPEP, IL6, KLF6, NDRG1, LTBP4, EREG, NRG1, ECGF1, ARNT2, NRP1, IFRD1
Behavior	11	0.004559090	CCL20, PLD1, CXCL2, APOE, MAOA, PI3, CXCL1, PLAU, IL8, ECGF1, CCL5
Angiogenesis	7	0.001885665	ANPEP, ANGPTL4, EREG, RHOB, IL8, ECGF1, NRP1
Nucleosome assembly	7	0.007444314	HIST1H2BD, HIST2H2AA, HIST2H2BE, H2BFS, HIST1H1C, HIST1H2BK, SLC22A4
Cell adhesion	24	0.00542196	CLDN12, CTGF, LAMA4, CSPG2, RHOB, PKD1, CLDN1, TNFAIP6, CCL5, TPBG, KIAA0319, APP, ITGA5, LPP, GPR56, VTN, NID2, MYO10, IL8, COL7A1, MSLN, NRP1, ITGA3, ITGA2
Morphogenesis	27	8.06E-05	IGF2BP3, IGFBP5, CTGF, ANGPTL4, APOE, EPC1, MSX1, KLF6, RRAGC, SLC3A2, WNT5A, IER3, ARHGEF2, DHRS9, PKD1, RHOB, VAT1, QSCN6, ANPEP, LTBP4, EREG, EDG3, IL8, DCN, ECGF1, NRP1, ITGA2
Taxis	8	0.009882707	CCL20, PLD1, CXCL2, CXCL1, PLAU, IL8, ECGF1, CCL5
Glutamine family amino acid metabolism	5	0.007880038	GLS, GCLC, GFPT2, ASS, ASNS

studies of insulin resistance[30]. A similar technique has also been utilized to induce growth suppression in human colon cancer cells, as reported by Sarraf *et al* (1998)[13]. This suggests that the clinical concentrations of troglitazone should effectively suppress the growth of stomach cancer cells.

The intracellular mechanisms relevant to the growth suppression effects of ciglitazone and troglitazone remain to be elucidated; however, Western blotting results have verified that ERK phosphorylation is suppressed by troglitazone and ciglitazone, and the noted increase in the manifestation of p21 is more marked with troglitazone. We conclude that ciglitazone and troglitazone are associated with the suppression of cell growth. Our microarray analysis results showed that troglitazone induces not only the expression

Table 3 Biological process of down-regulated genes by troglitazone in SNU-668 cells

Term	Count	*P* value	Genes
Mitotic sister chromatid segregation	10	1.61E-10	CDCA5, KNTC2, CNAP1, DLG7, NUSAP1, HCAP-G, SMC4L1, CENPE, SMC2L1, ESPL1
Phosphorylation	32	6.36E-04	CDK6, CCL2, BUB1, INHA, CDC7, PKMYT1, GSG2, PBK, BUB1B, CHEK1, LOC91461, MASTL, WEE1, AURKB, MXRA5, PKN3, VRK1, CIT, PRKCE, PLK2, MELK, PASK, TTK, NEK2, EME1, NUAK2, CDC2, CDK2, CDK4, MAP2K6, PLK1, PLK4
Mitotic chromosome condensation	6	2.73E-06	CDCA5, CNAP1, NUSAP1, HCAP-G, SMC4L1, SMC2L1
Mitosis	44	4.68E-37	KIF2C, CENPF, BUB1, SPAG5, DLG7, ACTG1, PKMYT1, CDC6, HCAP-G, MAD2L1, CCNB1, PBK, CCNF, BUB1B, ESPL1, CCNB2, WEE1, CNAP1, KIF23, SGOL1, CIT, CDC25A, TPX2, ASPM, SMC4L1, SMC2L1, MPHOSPH1, TTK, NEK2, CDC2, CDK2, CCNA2, CDC25C, PTTG1, KNTC1, KIF15, PLK1, CDCA5, CDC20, KNTC2, NUSAP1, ANLN, UBE2C, CENPE
Phosphoinositide-mediated signaling	13	8.33E-08	CKS2, RFC4, HMGB2, HIST1H4C, TYMS, SPAG5, BUB1B, TOP2A, KNTC2, ZWINT, PCNA, UBE2C, FEN1
Response to DNA damage stimulus	43	1.78E-25	RFC4, CHAF1B, FANCL, POLD3, NUDT1, UHRF1, EXO1, CHEK1, LIG1, BRCA1, TOP2A, POLE2, RAD54L, GTSE1, POLE, PCNA, RAD51, MDC1, UIP1, RAD51AP1, HMGB2, RPA1, TYMS, POLQ, NEIL3, CCNA2, CHAF1A, PTTG1, MAP2K6, RFC5, FANCG, RPA3, FANCB, XRCC3, H2AFX, TOPBP1, RECQL4, BLM, RAD51C, POLD1, FANCD2, APEX2, FEN1
Traversing start control point of mitotic cell cycle	5	2.73E-06	CDC7, CDC6, CDC2, CDK2, CDC25C
Regulation of DNA replication	5	2.14E-04	GMNN, CDC6, CDK2, CDT1, PCNA
Regulation of DNA metabolism	7	6.56E-05	BRCA1, GMNN, CDC6, CDK2, CDT1, PCNA, RAD51
Phosphate metabolism	35	1.83E-03	CDK6, CCL2, BUB1, INHA, CDC7, PKMYT1, GSG2, CDKN3, PBK, BUB1B, CHEK1, LOC91461, MASTL, WEE1, AURKB, MXRA5, PKN3, VRK1, CIT, CDC25A, PRKCE, MELK, PLK2, PASK, TTK, NEK2, EME1, NUAK2, CDC2, CDK2, CDC25C, CDK4, MAP2K6, PLK4, PLK1
Organelle organization and biogenesis	44	7.49E-08	CKS2, KRT8, PRC1, KIF2C, CENPF, SUV39H1, CHAF1B, SPAG5, EZH2, KIF14, HCAP-G, KIF4A, DIAPH3, BUB1B, ESPL1, BRCA1, ACD, HIST1H2BH, CNAP1, KIF23, CBX1, ZWINT, GTSE1, KIF11, SMC4L1, SMC2L1, EXOSC2, MPHOSPH1, STMN1, TTK, HMGB2, HIST1H4C, CENPA, KIF20A, CHAF1A, KIF15, CDCA5, H2AFX, KNTC2, MGC39900, NUSAP1, UBE2C, CENPE, C9orf48
Organelle localization	6	1.62E-06	CENPF, CDCA5, DLG7, NUSAP1, CENPE, ESPL1
Microtubule-based process	22	7.74E-11	STMN1, CKS2, PRC1, KIF2C, TTK, SPAG5, KIF14, KIF4A, KIF20A, BUB1B, ESPL1, KIF15, KNTC2, KIF23, NUSAP1, ZWINT, GTSE1, KIF11, UBE2C, CENPE, C9orf48, MPHOSPH1
Sister chromatid segregation	10	2.68E-10	CDCA5, KNTC2, CNAP1, DLG7, NUSAP1, HCAP-G, SMC4L1, CENPE, SMC2L1, ESPL1
Regulation of cyclin dependent protein kinase activity	10	1.66E-07	CHEK1, CKS2, CDK5RAP3, PKMYT1, BCCIP, CDC6, CDC25A, CDKN3, CCNA2, CDC25C
Deoxyribonucleotide biosynthesis	3	6.19E-03	RRM2, TYMS, DTYMK
Response to stress	56	1.70E-09	CCL2, EXO1, LIG1, RAD54L, BST1, GTSE1, POLE, MDK, TYMS, POLQ, NEIL3, CCNA2, PTTG1, RFC5, FANCG, RPA3, FANCB, XRCC3, H2AFX, RECQL4, HSPA2, FANCD2, APEX2, RFC4, CHAF1B, INHA, FANCL, POLD3, NUDT1, UHRF1, FOS, CHEK1, BRCA1, TOP2A, GP1BB, POLE2, PCNA, RAD51, MDC1, UIP1, RAD51AP1, FOXM1, HMGB2, RPA1, CHAF1A, MAP2K6, CFH, TOPBP1, CD14, NR3C1, BLM, CLEC2D, RAD51C, POLD1, FEN1, PRDX2
Metaphase plate congression	3	2.55E-03	CENPF, CDCA5, CENPE
Regulation of transferase activity	12	1.44E-04	CHEK1, CKS2, CDK5RAP3, TPD52L1, PKMYT1, BCCIP, CDC6, CDC25A, CDKN3, CCNA2, CDC25C, RGS4
DNA strand elongation	3	4.18E-03	PRIM1, RFC4, RFC3
Cell proliferation	37	9.94E-10	CKS2, CDK6, KIF2C, CENPF, BUB1, CDC7, SKP2, DLG7, CDC6, CDKN3, DTYMK, BUB1B, CHEK1, STIL, BRCA1, CDKN2C, CDC25A, TPX2, PCNA, IFITM1, TTK, CDK5RAP3, MDK, MKI67, CDK2, CDC25C, ADAMTS1, TSPAN3, CDK4, CDCA7, CYR61, KIF15, CDCA7L, HDGF, PLK1, E2F1, UBE2C
Establishment of organelle localization	6	4.57E-07	CENPF, CDCA5, DLG7, NUSAP1, CENPE, ESPL1
Cell organization and biogenesis	59	6.71E-06	PRC1, KIF2C, SUV39H1, DLG7, HCAP-G, DIAPH3, ESPL1, ACD, CNAP1, KIF23, CBX1, THOC4, KIF11, GTSE1, EXOSC2, MPHOSPH1, STMN1, IL17RB, CENPA, CYR61, H2AFX, KNTC2, NUSAP1, HNRPA1, C9orf48, SLC25A10, NUP107, CKS2, KRT8, CENPF, CHAF1B, SPAG5, EZH2, KIF14, KIF4A, BUB1B, BRCA1, HIST1H2BH, TRIP6, ZWINT, SMC4L1, SMC2L1, TTK, KAZALD1, HMGB2, HIST1H4C, RANBP1, IGFBP3, TMEM97, KIF20A, CHAF1A, SORT1, KIF15, PPIH, CDCA5, MGC39900, UBE2C, WISP2, CENPE
Regulation of kinase activity	12	1.35E-04	CHEK1, CKS2, CDK5RAP3, TPD52L1, PKMYT1, BCCIP, CDC6, CDC25A, CDKN3, CCNA2, CDC25C, RGS4
DNA repair	40	3.75E-24	RFC4, CHAF1B, FANCL, POLD3, NUDT1, UHRF1, EXO1, CHEK1, LIG1, BRCA1, TOP2A, POLE2, RAD54L, POLE, PCNA, RAD51, MDC1, UIP1, RAD51AP1, HMGB2, RPA1, TYMS, POLQ, NEIL3, CHAF1A, PTTG1, RFC5, FANCG, RPA3, FANCB, XRCC3, H2AFX, TOPBP1, RECQL4, BLM, RAD51C, POLD1, FANCD2, APEX2, FEN1
DNA recombination	11	3.91E-06	CHEK1, LIG1, XRCC3, H2AFX, RPA1, BLM, RAD51C, RAD54L, RAD51, RAD51AP1, EXO1

Protein complex assembly	14	7.55E-03	CENPF, HMGB2, CHAF1B, HIST1H4C, SLC7A6, MPP2, CENPA, CHAF1A, KNTC1, PPIH, H2AFX, HIST1H2BH, RAD51, CENPE
Establishment of chromosome localization	4	8.79E-05	CENPF, CDCA5, DLG7, CENPE
Chromosome segregation	15	1.42E-14	CENPF, DLG7, HCAP-G, PTTG1, SGOL2, ESPL1, CDCA5, KNTC2, CNAP1, SGOL1, NUSAP1, CDCA1, SMC4L1, SMC2L1, CENPE
Chromosome organization and biogenesis	19	9.65E-05	CENPF, SUV39H1, HMGB2, CHAF1B, HIST1H4C, EZH2, HCAP-G, CENPA, CHAF1A, CDCA5, ACD, H2AFX, CNAP1, HIST1H2BH, CBX1, NUSAP1, SMC4L1, SMC2L1, CENPE
DNA integrity checkpoint	6	2.66E-05	CHEK1, CDC6, GTSE1, CDT1, CCNA2, CDC45L
Chromosome localization	4	8.79E-05	CENPF, CDCA5, DLG7, CENPE
Spindle organization and biogenesis	11	4.01E-12	CKS2, STMN1, PRC1, TTK, KNTC2, SPAG5, KIF23, ZWINT, KIF11, UBE2C, BUB1B
Protein amino acid phosphorylation	30	8.41E-05	CDK6, CCL2, BUB1, CDC7, PKMYT1, GSG2, PBK, BUB1B, LOC91461, MASTL, CHEK1, WEE1, AURKB, MXRA5, PKN3, VRK1, CIT, PRKCE, PLK2, MELK, PASK, TTK, NEK2, NUAK2, CDC2, CDK2, CDK4, MAP2K6, PLK1, PLK4
Chromosome condensation	6	9.81E-06	CDCA5, CNAP1, NUSAP1, HCAP-G, SMC4L1, SMC2L1
Cell cycle	97	2.18E-51	PRC1, KIF2C, CDC7, GSG2, CDKN3, HCAP-G, ESPL1, LIG1, MCM5, TPD52L1, CNAP1, KIF23, RAD54L, GTSE1, STMN1, NEK2, E2F2, CDK4, SGOL2, KNTC1, PLK1, H2AFX, CKS1B, BCCIP, NUSAP1, ANLN, CKS2, CENPF, CHAF1B, ACTG1, CDC6, PBK, CHEK1, ZWINT, ASPM, RAD51, MDC1, SMC4L1, TTK, MKI67, CDK2, MCM7, KIF15, MAP2K6, CDCA5, E2F1, BIRC5, UBE2C, BUB1, PKMYT1, ILF3, DLG7, DTYMK, MAD2L1, WEE1, SGOL1, CIT, KIF11, RBL1, MPHOSPH1, MDK, CCNA2, CDT1, PTTG1, CDC45L, FANCG, PLK4, MCM3, KNTC2, GMNN, HSPA2, FANCD2, CDK6, INHA, SKP2, SPAG5, UHRF1, CCNB1, CCNF, BUB1B, CCNB2, BRCA1, CDKN2C, AURKB, TPX2, CDC25A, PCNA, SMC2L1, IFITM1, CDK5RAP3, MCM2, CDC2, CDC25C, MCM6, CHAF1A, CDC20, CENPE
Spindle checkpoint	3	4.18E-03	TTK, CENPF, BUB1
Cytoskeleton organization and biogenesis	25	4.74E-06	CKS2, KIF2C, PRC1, KRT8, SPAG5, KIF14, KIF4A, DIAPH3, BUB1B, ESPL1, KIF23, KIF11, GTSE1, ZWINT, MPHOSPH1, STMN1, TTK, KIF20A, KIF15, KNTC2, NUSAP1, MGC39900, UBE2C, C9orf48, CENPE
Chromatin assembly or disassembly	9	8.89E-03	SUV39H1, HIST1H4C, CHAF1B, HMGB2, H2AFX, HIST1H2BH, CBX1, CENPA, CHAF1A
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	121	1.16E-08	SUV39H1, Pfs2, ADARB1, ATOH8, CDC7, TAF5, CITED4, TK1, HMG1L1, EXO1, PRIM1, LIG1, MCM5, TPD52L1, ATPIF1, PAPSS2, CBX1, RAD54L, POLE, ORC6L, EXOSC2, IQGAP3, RRM2, ORC1L, TRIP13, E2F2, POLQ, ITGB3BP, CENPA, NEIL3, CHTF18, RRM1, KNTC1, RNASEH2A, ID3, RFC5, FANCB, XRCC3, H2AFX, GATA2, HNRPA1, DMBX1, NASP, RFC4, RFC2, CENPF, CHAF1B, PAICS, POLD3, CDC6, SREBF1, CHEK1, GNE, MCM4, MXD3, RAD51, MDC1, UIP1, RAD51AP1, TIMELESS, FOXM1, EME1, SLBP, HIST1H4C, CDK2, HAT1, MCM7, NR3C1, DNMT1, BLM, RAD51C, E2F1, POLD1, FEN1, ILF3, SNRPA, DTYMK, RAB26, ZNF488, THOC4, RBL1, TYMS, RPA2, CDT1, PTTG1, CDC45L, FANCG, RPA3, HOXA2, GMNN, MCM3, RECQL4, FANCD2, POLA2, APEX2, FANCL, EZH2, NUDT1, PHF19, UHRF1, FOS, POLR3K, BRCA1, TOP2A, ASCC3L1, HIST1H2BH, POLE2, PCNA, NUDT21, HMGB2, RPA1, MCM2, MCM6, CHAF1A, PPIH, SLC2A4RG, TOPBP1, RFC3, TTF2, C20orf129, CSTF3
Second-messenger-mediated signaling	16	1.53E-05	APITD1, CKS2, RFC4, GABBR2, CCL2, HMGB2, HIST1H4C, TYMS, SPAG5, BUB1B, TOP2A, KNTC2, ZWINT, PCNA, UBE2C, FEN1
Meiosis	7	4.68E-04	CHEK1, NEK2, H2AFX, SGOL1, RAD54L, HSPA2, RAD51
Regulation of protein kinase activity	12	1.35E-04	CHEK1, CKS2, CDK5RAP3, TPD52L1, PKMYT1, BCCIP, CDC6, CDC25A, CDKN3, CCNA2, CDC25C, RGS4

Table 4 Pathway of down-regulated genes by troglitazone in SNU-668 cells

Term	Count	*P* value	Genes
DNA Polymerase	8	2.37E-06	PRIM1, RFC5, POLD3, POLE2, POLQ, POLD1, POLE, POLA2
Pyrimidine metabolism	13	6.71E-06	RRM2, TYMS, POLD3, TK1, DTYMK, RRM1, POLR3K, PRIM1, RFC5, POLE2, POLD1, POLE, POLA2
Purine metabolism	14	2.64E-04	RRM2, PAICS, POLD3, PDE7B, RRM1, POLR3K, PRIM1, RFC5, PDE4B, PAPSS2, POLE2, POLD1, POLE, POLA2
Cell cycle	34	3.15E-27	CDK6, BUB1, CDC7, SKP2, PKMYT1, CDC6, MAD2L1, CCNB1, BUB1B, ESPL1, CCNB2, CHEK1, WEE1, MCM5, MCM4, CDKN2C, CDC25A, PCNA, ORC6L, RBL1, ORC1L, MCM2, CDC2, CDK2, MCM6, CDC25C, CCNA2, CDK4, PTTG1, CDC45L, MCM7, PLK1, CDC20, MCM3

of p21-inducing cell-cycle-controlling genes, but also suppresses expression of genes associated with DNA composition and a variety of other genes. This suggests that transcription of many crucial genes is completely unrelated to PPAR-γ in the presence of troglitazone. As shown above, the growth-suppressive effects induced by ciglitazone and triglitazone occur *via* a PPAR-independent pathway, and transcription of a variety of genes associated with the induction of cell-cycle control and DNA compound factors are relevant to this process.

COMMENTS

Background

Peroxisome proliferation-activated receptor (PPAR)-γ is manifested in a variety

of tissues and cancer cells and the ligands that activate PPAR-γ are currently being studied as a novel treatment. The PPAR-γ ligand generally decreases the survival rate of cancer cells *via* differentiation, apoptotic induction, and changes in genes or proteins associated with entrance into the G1/S phase. We studied the appearance of PPAR-γ in two types of stomach cancer cells treated with ciglitazone and troglitazone, both of which induce PPAR-γ activation. We were able to identify cell proliferation, confirm the effects of PPAR-γ suppressors, and clarify any genetic alterations for the growth of stomach cancer cells using cDNA microarrays.

Research frontiers

They evaluated the effects of PPAR-γ and PPAR-γ agonists on stomach cancer cells at the cytological and molecular levels, and determined the concentration of troglitazone that can be used clinically to suppress the growth of stomach cancer cells. In 1999, Takahashi *et al* reported that stomach cancer is suppressed by PPAR-γ-ligand-mediated apoptosis. In 2005, Lu *et al* reported that troglitazone suppresses stomach cancer *via* the activation of PPAR-γ.

Innovations and breakthroughs

This manuscript shows a growth suppressing effect of the PPAR-γ ligands on stomach cancer cells *via* a pathway independent of PPAR-γ activation.

Applications

Currently, PPAR-γ is used to treat diabetes mellitus, hyperlipidemia, atherosclerosis, inflammatory vascular disease, Alzheimer's disease and some malignant diseases. In particular, the suppressing effect of the PPAR-γ ligands on stomach cancer cells may contribute to treatment efficacy.

Terminology

PPAR is a member of the family of nuclear receptors, which is part of the steroid/thyroid hormone/retinoid receptor superfamily. PPAR has three subtypes, α, β/δ, and γ. PPAR-γ has novel functions in cell division and differentiation, which are associated with inflammatory response, tissue resuscitation, vascular biology, and cancer formation, as a control factor for apoptosis.

Peer review

This manuscript describes a growth-suppressing effect of the PPAR-γ ligands on stomach cancer cell line SNU-668, but not SNU-216. This effect is independent of PPAR-γ activation. Associated with this, was an increase in p21 and decreased phosphorylated-ERK. The authors then went on to perform microarray analysis after treatment with PPAR-γ ligands.

REFERENCES

1 **Chawla A**, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. *Science* 2001; **294**: 1866-1870

2 **Han S**, Roman J. Peroxisome proliferator-activated receptor gamma: a novel target for cancer therapeutics? *Anticancer Drugs* 2007; **18**: 237-244

3 **Elangbam CS**, Tyler RD, Lightfoot RM. Peroxisome proliferator-activated receptors in atherosclerosis and inflammation--an update. *Toxicol Pathol* 2001; **29**: 224-231

4 **Lehrke M**, Lazar MA. The many faces of PPARgamma. *Cell* 2005; **123**: 993-999

5 **Elstner E**, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, Shintaku P, Said JW, Heber D, Koeffler HP. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. *Proc Natl Acad Sci USA* 1998; **95**: 8806-8811

6 **Ohta K**, Endo T, Haraguchi K, Hershman JM, Onaya T. Ligands for peroxisome proliferator-activated receptor gamma inhibit growth and induce apoptosis of human papillary thyroid carcinoma cells. *J Clin Endocrinol Metab* 2001; **86**: 2170-2177

7 **Rumi MA**, Sato H, Ishihara S, Kawashima K, Hamamoto S, Kazumori H, Okuyama T, Fukuda R, Nagasue N, Kinoshita Y. Peroxisome proliferator-activated receptor gamma ligand-induced growth inhibition of human hepatocellular carcinoma. *Br J Cancer* 2001; **84**: 1640-1647

8 **Heaney AP**, Fernando M, Melmed S. PPAR-gamma receptor ligands: novel therapy for pituitary adenomas. *J Clin Invest* 2003; **111**: 1381-1388

9 **Giaginis C**, Theocharis S, Tsantili-Kakoulidou A. A consideration of PPAR-gamma ligands with respect to lipophilicity: current trends and perspectives. *Expert Opin Investig Drugs* 2007; **16**: 413-417

10 **Lu J**, Imamura K, Nomura S, Mafune K, Nakajima A, Kadowaki T, Kubota N, Terauchi Y, Ishii G, Ochiai A, Esumi H, Kaminishi M. Chemopreventive effect of peroxisome proliferator-activated receptor gamma on gastric carcinogenesis in mice. *Cancer Res* 2005; **65**: 4769-4774

11 **Takahashi N**, Okumura T, Motomura W, Fujimoto Y, Kawabata I, Kohgo Y. Activation of PPARgamma inhibits cell growth and induces apoptosis in human gastric cancer cells. *FEBS Lett* 1999; **455**: 135-139

12 **Brockman JA**, Gupta RA, Dubois RN. Activation of PPARgamma leads to inhibition of anchorage-independent growth of human colorectal cancer cells. *Gastroenterology* 1998; **115**: 1049-1055

13 **Sarraf P**, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, Spiegelman BM. Differentiation and reversal of malignant changes in colon cancer through PPARgamma. *Nat Med* 1998; **4**: 1046-1052

14 **Kitamura S**, Miyazaki Y, Shinomura Y, Kondo S, Kanayama S, Matsuzawa Y. Peroxisome proliferator-activated receptor gamma induces growth arrest and differentiation markers of human colon cancer cells. *Jpn J Cancer Res* 1999; **90**: 75-80

15 **Shimada T**, Kojima K, Yoshiura K, Hiraishi H, Terano A. Characteristics of the peroxisome proliferator activated receptor gamma (PPARgamma) ligand induced apoptosis in colon cancer cells. *Gut* 2002; **50**: 658-664

16 **Chen ZY**, Tseng CC. 15-deoxy-Delta12,14 prostaglandin J2 up-regulates Kruppel-like factor 4 expression independently of peroxisome proliferator-activated receptor gamma by activating the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase signal transduction pathway in HT-29 colon cancer cells. *Mol Pharmacol* 2005; **68**: 1203-1213

17 **Chintharlapalli S**, Papineni S, Baek SJ, Liu S, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substitutedphenyl)methanes are peroxisome proliferator-activated receptor gamma agonists but decrease HCT-116 colon cancer cell survival through receptor-independent activation of early growth response-1 and nonsteroidal anti-inflammatory drug-activated gene-1. *Mol Pharmacol* 2005; **68**: 1782-1792

18 **Chintharlapalli S**, Papineni S, Konopleva M, Andreef M, Samudio I, Safe S. 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid and related compounds inhibit growth of colon cancer cells through peroxisome proliferator-activated receptor gamma-dependent and -independent pathways. *Mol Pharmacol* 2005; **68**: 119-128

19 **Willson TM**, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. *J Med Chem* 2000; **43**: 527-550

20 **Dennis G Jr**, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 2003; **4**: P3

21 **Ikezoe T**, Miller CW, Kawano S, Heaney A, Williamson EA, Hisatake J, Green E, Hofmann W, Taguchi H, Koeffler HP. Mutational analysis of the peroxisome proliferator-activated receptor gamma gene in human malignancies. *Cancer Res* 2001; **61**: 5307-5310

22 **Desvergne B**, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 1999; **20**: 649-688

23 **Escher P**, Wahli W. Peroxisome proliferator-activated receptors: insight into multiple cellular functions. *Mutat Res* 2000; **448**: 121-138

24 **Murphy GJ**, Holder JC. PPAR-gamma agonists: therapeutic role in diabetes, inflammation and cancer. *Trends Pharmacol Sci* 2000; **21**: 469-474

25 **Fajas L**, Debril MB, Auwerx J. Peroxisome proliferator-activated receptor-gamma: from adipogenesis to carcinogenesis. *J Mol Endocrinol* 2001; **27**: 1-9

26 **Grommes C**, Landreth GE, Heneka MT. Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists. *Lancet Oncol* 2004; **5**: 419-429
27 **Takeuchi S**, Okumura T, Motomura W, Nagamine M, Takahashi N, Kohgo Y. Troglitazone induces G1 arrest by p27(Kip1) induction that is mediated by inhibition of proteasome in human gastric cancer cells. *Jpn J Cancer Res* 2002; **93**: 774-782
28 **Jung TI**, Baek WK, Suh SI, Jang BC, Song DK, Bae JH, Kwon KY, Bae JH, Cha SD, Bae I, Cho CH. Down-regulation of peroxisome proliferator-activated receptor gamma in human cervical carcinoma. *Gynecol Oncol* 2005; **97**: 365-373
29 **Baek SJ**, Wilson LC, Hsi LC, Eling TE. Troglitazone, a peroxisome proliferator-activated receptor gamma (PPAR gamma) ligand, selectively induces the early growth response-1 gene independently of PPAR gamma. A novel mechanism for its anti-tumorigenic activity. *J Biol Chem* 2003; **278**: 5845-5853
30 **Nolan JJ**, Ludvik B, Beerdsen P, Joyce M, Olefsky J. Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med* 1994; **331**: 1188-1193

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Therapeutic effects of four strains of probiotics on experimental colitis in mice

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Abstract

AIM: To investigate the therapeutic effects of four strains of probiotics (*E. feacalis*, *L. acidophilus*, *C. butyricum* and *B. adolescentis*) on dextran sulphate sodium (DSS)-induced experimental colitis in Balb/c mice.

METHODS: Eighty Balb/c mice were randomly divided into 8 groups. Weight-loss, fecal character, fecal occult blood and hematochezia were recorded daily. Disease activity index (DAI) scores were also evaluated everyday. Length of colon was measured and histological scores were evaluated on the 13th day. Myeloperoxidase (MPO) activity was detected. Interleukin-1 (IL-1) and IL-4 expression was detected by ELISA and RT-PCR.

RESULTS: The four strains of probiotics relieved the inflammatory condition of DSS-induced experimental colitis in mice. Weight loss was slowed down in all probiotics-treated mice. Even weight gain was observed by the end of probiotics treatment. The DAI and histological scores of probiotics-treated mice were lower than those of mice in the control group (1.9 ± 0.2 *vs* 8.6 ± 0.4, $P < 0.05$ for *E. faecalis*). The length of colon of probiotics-treated mice was longer than that of mice in the control group (10.3 ± 0.34 *vs* 8.65 ± 0.77, $P < 0.05$ for *E. faecalis*). The four strains of probiotics decreased the MP activity and the IL-1 expression, but increased the IL-4 expression. *E. faecalis* had a better effect on DSS-induced experimental colitis in mice than the other three strains.

CONCLUSION: The four strains of probiotics have beneficial effects on experimental colitis in mice. *E. faecalis* has a better effect on DSS-induced experimental colitis in mice than the other three strains. Supplement of probiotics provides a new therapy for UC.

Key words: Probiotic; *E. faecalis*; Experimental colitis; Interleukin-1; Interleukin-4

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INTRODUCTION

Ulcerative colitis (UC) is a non-specific chronic inflammation of intestinal tract. The primary therapies with salazosulfamide, glucocorticoids and immunodepressant probiotics are limited by their side-effects, poor compliance of patients and high relapse rates[1-3].

It is well known that consumption of certain bacteria improves intestinal health[4-6]. Animal and clinical studies also indicate that gastrointestinal bacteria play an important role in the development of UC[7,8]. Supplements of probiotics provide a new therapy for UC. Bibiloni *et al*[9] found that VSL#3 (including 4 strains of *Lactobacilli*, 3 strains of *Bifidobacteria* and 1 strain of *Streptococcus*) displays a beneficial effect on UC.

Kruis *et al*[10] showed that *Escherichia coli* and Nissle 1917 have a similar effect and safety on 5-aminosalicylic acid[10]. It was reported that administration of *Lactobacillus casei* obviously decreases histological damage, while administration of *Escherichia coli* O83 and Nissle 1917 is beneficial for immunological regulation[11]. Peran *et al*[12] showed that probiotics can relieve UC symptoms through different mechanisms of action[12].

Because of the specific damage site of UC and the different colonizations of each bacterium, different probiotics have different effects on UC[13-15]. In order to find out effective strains, we compared the effects of four strains of probiotics on experimental colitis in mice.

MATERIALS AND METHODS

Bacterial strains

L. acidophilus, C. butyricum, B. adolescentis and *E. feacalis* were isolated from intestinal tract of healthy adults and identified in our facility.

Reagents

Salicylazosulfapyridine (SASP) was purchased from Fuda Pharmaceutical Co. Ltd (China). Dextran sulphate sodium was produced by MW 5000, Sigma-Aldrich Co (USA). ELISA kit was bought from Boster Biological Technology, Ltd (China), RT-PCR correlate agents were purchased from Promega Biotech Co., Ltd (USA) and Myeloperoxidase (MPO) diagnostic kit was bought from Jiancheng biotech Co (China).

Experimental design

Six-eight-week-old Balb/c mice (half males and half females, weighing 20.0 ± 2.0 g) provided by Hunan Agricultural University (China) were randomly divided into 8 groups, housed in clean filter-top cages under standard conditions (50% ± 10% humidity) in a 12-h dark/12-h light cycle, and fed with standard mouse chow. All mice were fed under standard conditions for 5 d. All mice were divided as follows: (1) Normal group: Normal diet without special treatment; (2) Model group: drinking 5% DSS for modeling; (3) NS group: 5% DSS for modeling + NS (100 μL/10 g) by gavage (4) SASP group: 5% DSS for modeling + SASP (50 mg/mL) by gavage; (5) *L. acidophilus* group: 5% DSS for modeling + 10^9 U/mL *L. acidophilus* by gavage; (6) *C. butyricum* group: 5% DSS for modeling +10^9 U/mL *C. butyricum* by gavage; (7) *B. adolescentis* group: 5% DSS for modeling +10^9 U/mL *B. adolescentis* by gavage; (8) *E. faecalis* group: 5% DSS for modeling +10^9 U/mL *E. faecalis* by gavage; The four strains of probiotics were grown overnight in culture media and suspended in normal saline (NS) to a concentration of 10^9 U/mL. SASP was dissolved in NS to a concentration of 50 mg/mL. The mice were fed with 200 μL of this daily-prepared suspension *via* an intragastric tube. On the 13th day, all mice were sacrificed.

To reflect the general conditions of mice, DAI scores were determined by an investigator blind to the protocol by scoring the extent of body weight loss, fecal character, fecal occult blood or hematochezia as previously described[16]. On the 13th day, blood was collected and all mice were sacrificed. The colon, from the colo-cecal junction to the anus was excised with its length measured, rinsed with 5 mL of 0.01 mol/L PBS (pH 7.4) to remove the fecal remnants, cut open longitudinally at the mesenteric attachment. One cm of the distal colon was removed and fixed for 48 h in PBS-buffered 10% formalin. The tissue was then processed for paraffin embedding and cut into 5-μm thick sections. The sections were stained with hematoxylin and eosin (HE). Other part of the colon was preserved in liquid nitrogen.

Table 1 PCR primers and products

	Primer		Product (bp)
IL-1β	Sense:	5'-AGCCCATCCTCTGTGACTCATG-3'	422
	Antisense:	5'-GCTGATGTACCAGTTGGGGAAC-3'	
IL-4	Sense:	5'-ACTTCAGTGGCTGGATTTAT-3'	424
	Antisense:	5' ATTCCCTGAAAGGCTTGGTC-3'	
β-actin	Sense:	5' ATGGATGACGATATCGCT-3'	569
	Antisense:	5'-ATGAGGTAGTCTGTCAGGT-3'	

MPO activity

MPO activity was measured as an indicator of neutrophil accumulation in colonic mucosa as preciously described[17].

RT-PCR

All primers were designed by software Primer5 (Table 1). The anneal temperature was 58-53℃ for 45 min at 30 amplification (Table 1).

ELISA analysis

The concentrations of IL-1 and IL-4 were measured in homogenized colons with an ELISA kit according to its manufacturers protocol and expressed as per milligram of protein.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 13.0. All results were expressed as mean ± SD. Data sets were analyzed by one-way analysis of variance (ANOVA) and Fishers' protected LSD post *hoc*-test. Those with a significant difference were further analyzed by Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

Weight loss

No difference was observed in body weight of mice among the 4 groups on day 0 (ANOVA). Body weight increased gradually in the normal group. Mice in the model group had a weight gain during the first 4 d due to lack of

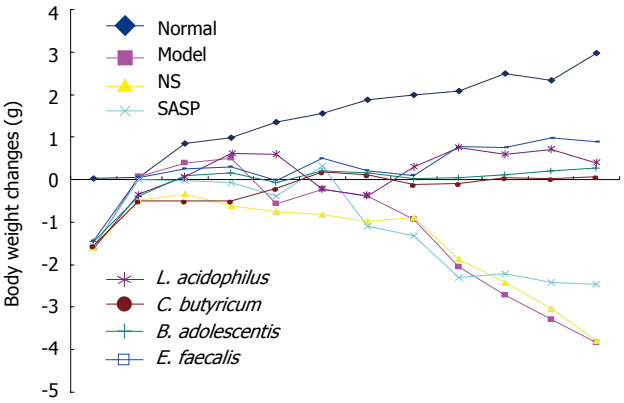

Figure 1 Body weight changes in all groups. The body weight of mice on the 1st day was taken as the basal level. The body weight of mice each day minus the basal body weight was expressed as the body weight change. The negative value indicates the decreased weight, and the positive value indicates the increased weight.

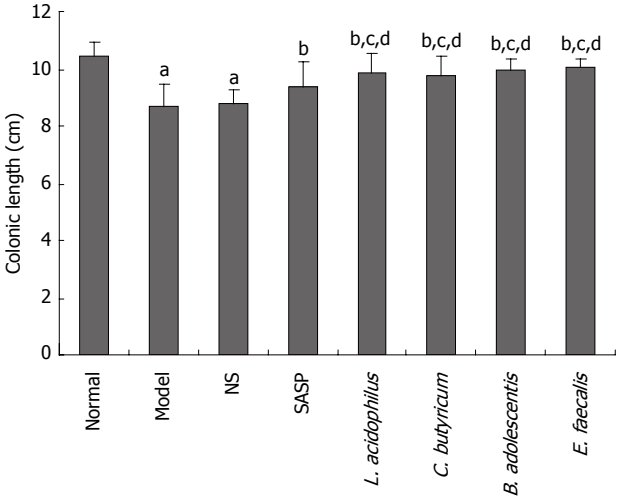

Figure 2 Length of colon on the 13th day. [a]$P < 0.05$ *vs* normal group; [b]$P < 0.05$ *vs* model group; [c]$P > 0.05$ *vs* normal group; [d]$P < 0.05$ *vs* SASP treatment group.

gavage stimulation, and a significant weight loss from day 5. Because of the double stimulation of DSS and gavages, mice in the NS group had an obviously weight loss. Mice in the SASP group maintained their body weight at its base level during the first 7 d, but had a significant weight loss from day 8 due to severe experimental colitis. The four strains of probiotics, especially *E. feacalis*, could inhibit the weight loss (Figure 1).

Length of colon

The length of colon of mice in the model and NS groups was significantly decreased compared with the SASP group. C. *butyricum*, L. *acidophilus*, *B. adolescentis*, *E. faecalis* could effectively prevent the shortening of colon (Figure 2).

DAI scores

Weight loss, fecal character, fecal occult blood and hematochezia were evaluated individually as previously described[16]. The highest DAI score was observed in

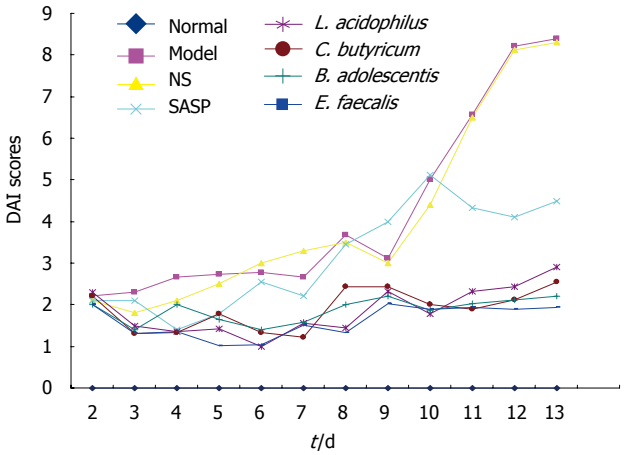

Figure 3 DAI scores of different groups. The DAI score was zero in normal group. The score increased gradually and reached 8.6 on the 13th day in model group. The score was low in SASP treatment group during the first 8 d, increased gradually during the last 5 d and reached 5.0 on the 13th day. The DAI scores of all four strains of probiotics were low in probiotics treatment groups, especially in *E. faecalis* treatment group. The maximum DAI score was only 1.9.

model and NS groups. SASP had a good effect on early experimental colitis, but a poor long-term effect on severe experimental colitis. The DAI scores of the four groups were low (Figure 3).

Histological scores

Histological changes in mice of the 4 groups were evaluated individually as previously described[18] (Figure 4). The highest score was found in mice of the model and NS groups, and a lower score was observed in mice of the SASP and probiotics treatment groups compared to mice of the model group, especially the mice in *E. faecalis* treatment group (1.67 ± 0.27 *vs* 9.99 ± 1.48, $P < 0.05$) (Figure 5).

MPO activity

The level of MPO activity was low in normal group, high in model group, and lower in SASP and probiotics treatment groups than in model group, especially in *E. faecalis* treatment group (Figure 6).

RT-PCR

The level of IL-1β mRNA was the lowest in normal group, the highest in model group, and lower in SASP and probiotics treatment groups than in model group (0.82 ± 0.03 *vs* 1.78 ± 0.07, $P < 0.05$) (Figure 7A-B). On the contrary, the level of IL-4 mRNA was the highest in normal group, the lowest in model group, higher in SASP and probiotics treatment groups than in model group (0.98 ± 0.01 *vs* 0.30 ± 0.01, $P < 0.05$) (Figure 8A-B)

ELISA analysis

The level of IL-1β and IL-4 was similar with that of mRNA. The level of IL-1β was the lowest *in L. acidophilus* and *E. faecalis* treatment groups (105.25 ± 7.79 *vs* 166.93 ± 13.69, $P < 0.05$), and the highest *in L. acidophilus*, *B. adolescentis*, and *E. faecalis* treatment groups (184.85 ± 11.51 *vs* 119.33 ± 10.86, $P < 0.05$) (Table 2).

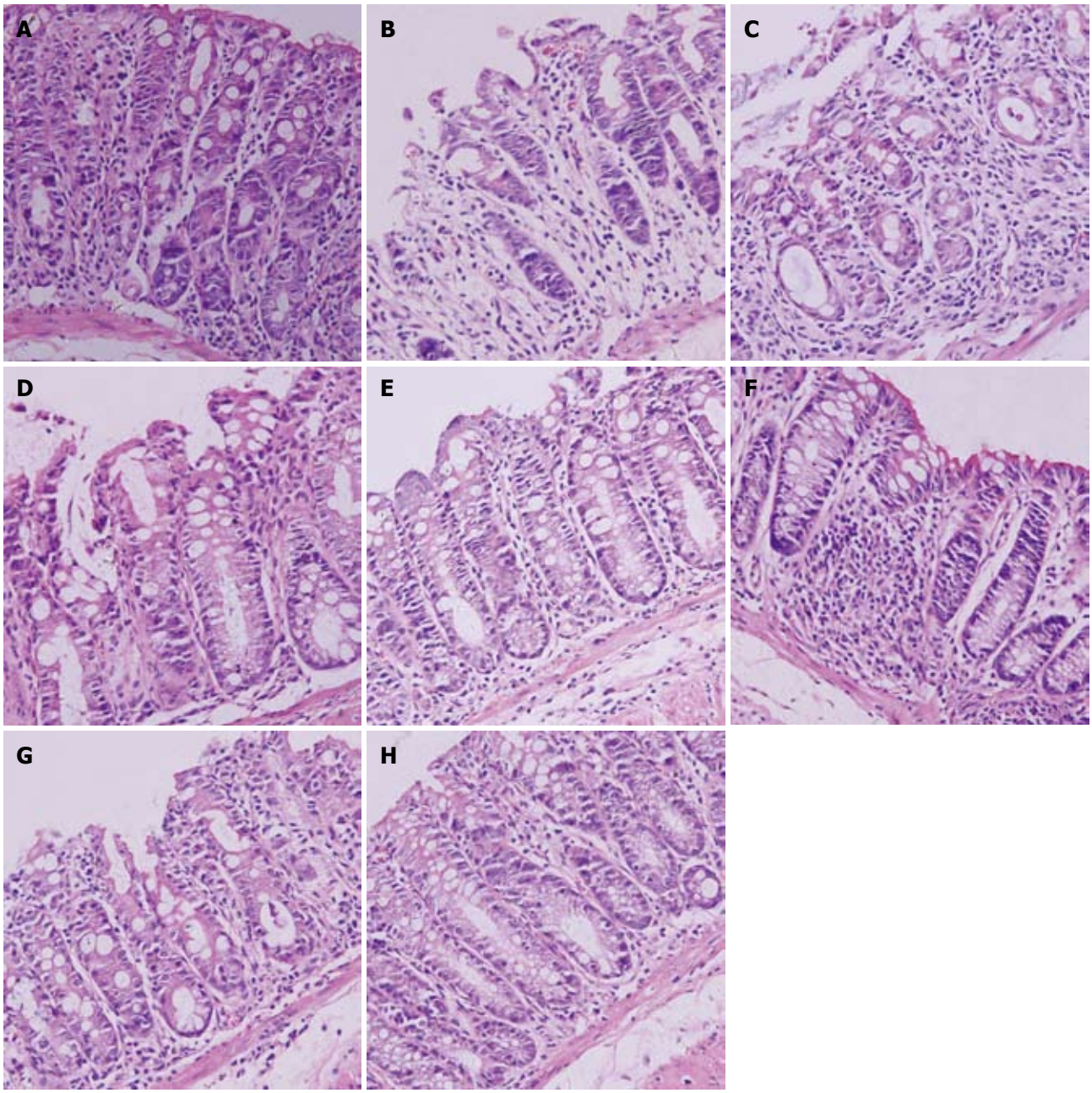

Figure 4 Histological images of mice. A: Normal group; B: Model group;C: NS group; D: SASP treatment group, E: *L. acidophilus* treatment group, F: *C. butyricum* treatment group, G: *B. adolescentis* treatment group, H: *E. faecalis* treatment group (HE, light microscope, x 200).

DISCUSSION

Studies showed that intestinal bacteria play an important role in the development of UC[5,19], and supplement of probiotic is beneficial for UC[13,14]. Because the damage site of UC resides mostly at the colon or rectum and different bacteria have different secretion functions or metabolism in intestinal tract, complement of local bacteria is more beneficial for UC. There are 300-500 different species of bacteria in the intestinal tract[5], and their location remains unclear at present. Since probiotics isolated from intestinal tract have an excellent permanent planting ability, obviously effective probiotics should be selected in a comparative study about their effects on experimental colitis.

Several studies on *Escherichia coli* Nissle, *Lactobacillus casei*, *Bifidobacterium lactis, Lactobacillus acidophilus, etc*, showed that these probiotics can be used in treatment of inflammatory bowel disease (IBD)[11,12,20]. In the present study, the effects of the four strains of probiotics on experimental colitis were compared.

The results of our study indicate that the four strains of probiotics had therapeutic effects on experimental colitis, confirming that probiotics can be used in treatment of colitis. Weight loss was slowed down and even weight gain was observed by the end of our experiment. The DAI and histological scores were low in probiotics treatment groups. These results agree with the reported findings[13,21]. The MPO activity decreased significantly in all probiotics treatment groups,

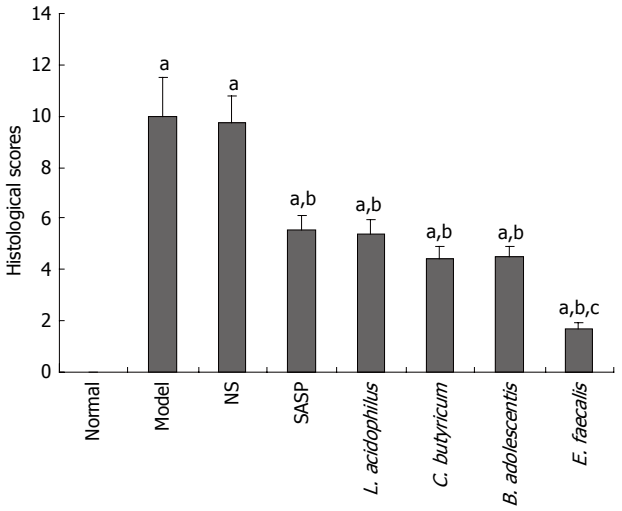

Figure 5 Histological scores of mice in different groups (mean ± SD, *n* = 10). The histological score was zero in normal group was zero ($^aP < 0.0$). The highest score was 9.9 ± 1.50 in model and NS groups ($^bP < 0.05$). The score was different in *E. faecalis* and SASP treatment groups ($^cP < 0.05$).

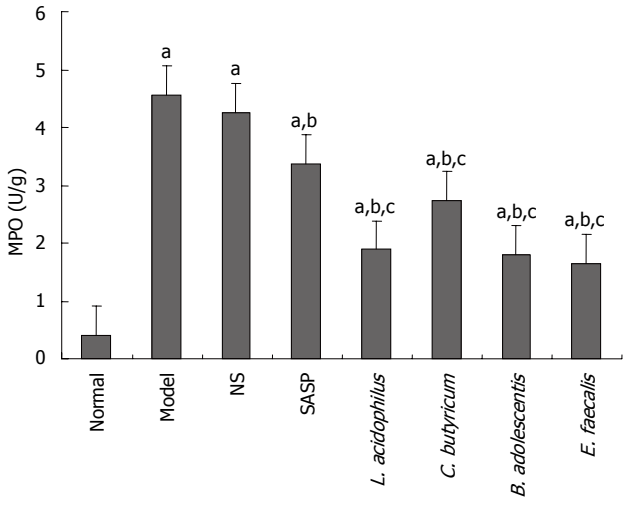

Figure 6 MPO activity in normal group (0.399 ± 0.133 U/g compared with normal group, model group and SASP treatment group. $^aP < 0.05$ *vs* normal group, $^bP < 0.05$ *vs* model group, $^cP < 0.05$ *vs* SASP treatment group.

Table 2 Concentrations of IL-1β and IL-4 used in ELISA analysis (mean ± SD)

Groups	Number	IL-1β (pg/mg.protein)	IL-4 (pg/mg.protein)
Norml	10	84.64 ± 7.02	205.81 ± 14.83
Model	10	166.93 ± 13.69[a]	119.33 ± 10.86[a]
NS	10	147.36 ± 12.61[a]	124.37 ± 9.85[a]
SASP	10	125.1 ± 12.04[a,c]	147.02 ± 12.02[a,c]
DSS + *L. acidophilus*	10	109.43 ± 11.98[a,c,e]	177.81 ± 10.29[a,c,e]
DSS + *C. butyricum*	10	121.25 ± 12.42[a,c,e]	150.76 ± 9.98[a,c]
DSS + *B. adolescentis*	10	120.27 ± 10.90[a,c]	173.69 ± 11.98[a,c,e]
DSS + *E. faecalis*	10	105.25 ± 7.79[a,c,e]	184.85 ± 11.51[c,e]

$^aP < 0.05$ *vs* normal group; $^cP < 0.05$ *vs* model group; $^eP < 0.05$ *vs* SASP group.

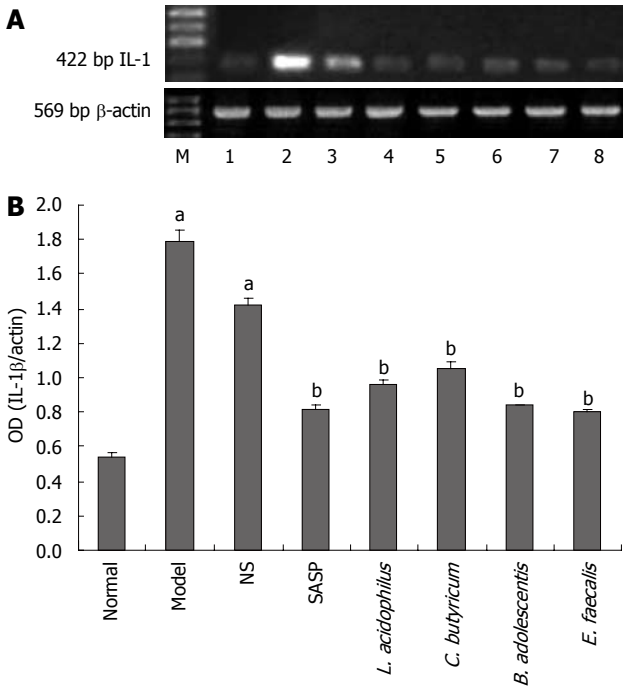

Figure 7 A-B Level of IL-1 βmRNA. M: Marker; 1: Normal group; 2: Model group; 3: NS group; 4: SASP treatment group; 5: *L. acidophilus* treatment group; 6: *C. butyricum* treatment group; 7: *B. adolescentis* treatment group; 8: *E. faecalis* treatment group. $^aP < 0.05$ *vs* normal group; $^bP < 0.05$ *vs* model group.

especially in *E. faecalis* treatment group, suggesting that the four stains of probiotics can relieve the symptoms of experimental colitis by decreasing the infiltration of neutrophils.

Balish *et al*[22] revealed that IBD occurs in germ-free IL-10-/- mice when they are colonized with a pure culture of *E. faecalis*, indicating *E. faecalis* is a conditioned pathogen. However, our study showed that *E. faecalis* could relieve the symptoms of experimental colitis and decrease the infiltration of neutrophils. *E. faecalis* had a better effect on experimental colitis than the other three strains of probiotics. Ruiz *et al*[23] reported that the expression of pro-inflammatory cells is transient 1 wk after *E. faecalis* treatment in intestinal epithelial cells from wild-type mice, suggesting that lack of protective TGF-β/Smad signaling and failing to inhibit TLR2-mediated pro-inflammatory gene expression in the intestinal epithelium might be a partial mechanism of IBD developed in IL-10-/- mice.

Because *E. faecalis*, *L. acidophilus* and some other probiotics have different effects on experimental colitis in wild-type mice rather than in immunodeficient mice, there should be more immunological mechanisms against experimental colitis except for the signal pathway or innate immunological mechanism. Some inflammatory cytokines, especially IL-1 and IL-4, are closely related with the development and progress of experimental colitis and IBD. IL-1 is secreted by mononuclear macrophages and high doses of IL-1, especially IL-1β, can result in UC[24]. IL-4 is synthesized by lamina propria intestinal lymphocytes after *in vitro* polyclonal activation. Compared with peripheral lymphocytes, intestinal epithelial and lamina propria lymphocytes spontaneously secrete IL-4[25], which can inhibit secretion of IL-1β by monocytes in a dose-dependent manner[26], suggesting

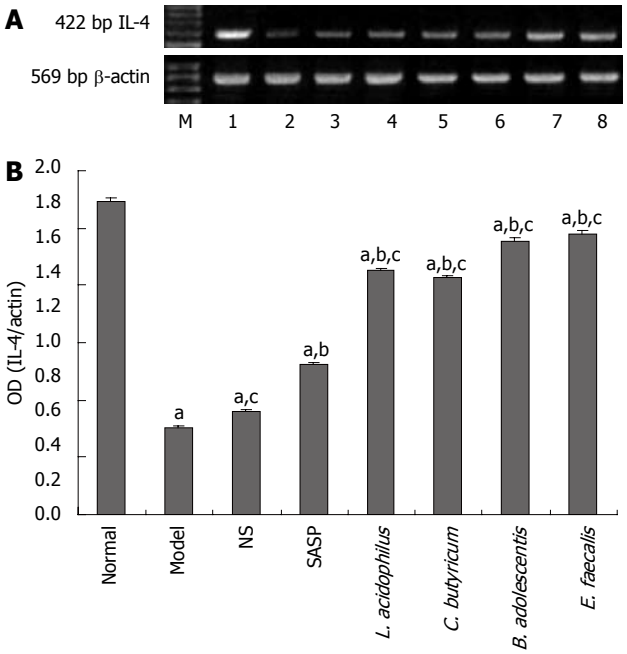

Figure 8 A-B Level of IL-4 mRNA. M: Marker; 1: Normal group; 2: Model group; 3: NS group; 4: SASP treatment group; 5: *L. acidophilus* treatment group; 6: *C. butyricum* treatment group; 7: *B. adolescentis* treatment group; 8: *E. faecalis* treatment group. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.05$ *vs* model group; $^{c}P < 0.05$ *vs* SASP treatment group.

that IL-4 has a protective function on regulatiing immunological reaction in intestinal tract. Our study showed that the expression of IL-1β was lower in probiotics treatment groups than in model group, and similar to that in SASP treatment group. The expression of IL-4 in probiotics treatment groups was increased. The expression of IL-4 was higher in *E. feacalis* treatment group than in treatment SASP group. The general condition, DAI scores, histological scores, and MPO activity were maintained at a parallel level, supporting the effects of probiotics at cytokine level. Changes in IL-1 and IL-4 level represent the inflammation degree of experimental colitis. Our experimental results also indicate that there should be some other inflammatory cytokines involved in the difference of adaptive immunological mechanisms in experimental colitis of wide-type and immunodeficient mice.

In summary, *L. acidophilus, C. butyricum, B. adolescentis, E. faecalis* are effective against DSS-induced acute experimental colitis. Reduced infiltration of neutrophils, decreased expression of IL-1 and increased expression of IL-4 might be a partial immunological mechanism of probiotics on experimental colitis in mice.

COMMENTS

Background

Ulcerative colitis (UC) is a non-specific chronic inflammation of intestinal tract and the primary therapies with probiotics are limited by their side-effects, poor compliance of patients and high relapse rates. Supplement of probiotics provides a new therapy for UC.

Research frontiers

Bacteria play an important role in pathogenesis of UC. Supplement of probiotics provide a new therapy for UC. Because of the specific damage site of UC and the different colonization sites of bacteria, different probiotics display different effects on UC. A more effective strain of probiotics should be selected for UC.

Innovations and breakthroughs

They compared the effects of four strains of probiotics isolated from healthy human feces in order to find one or two more effective strains. *E. faecalis* had a better effect than the other three strains. Their study showed that *E. faecalis* had different effects on experimental colitis in wild-type mice. The experimental results indicate that there should be some other inflammatory cytokines involved in experimental colitis of wide-type and immunodeficient mice except for IL-1 and IL-4.

Applications

The results of their study indicate that there should be some other inflammatory cytokines involved in experimental colitis of wide-type and immunodeficient mice except for IL-1 and IL-4.

Peer review

The effects of four strains of probiotics on DSS-induced colonic inflammation in mice were clarified. The authors showed that the four strains of probiotics derived from healthy human feces could relieve colonic inflammation as sulfasalazine. Of the four strains, *E. faecalis* was most beneficial for DSS-induced colitis. Methods employed and results obtained are reasonable.

REFERENCES

1 **Regueiro M**, Curtis J, Plevy S. Infliximab for hospitalized patients with severe ulcerative colitis. *J Clin Gastroenterol* 2006; **40**: 476-481
2 **Jakobovits SL**, Jewell DP, Travis SP. Infliximab for the treatment of ulcerative colitis: outcomes in Oxford from 2000 to 2006. *Aliment Pharmacol Ther* 2007; **25**: 1055-1060
3 **Lewis JD**, Gelfand JM, Troxel AB, Forde KA, Newcomb C, Kim H, Margolis DJ, Strom BL. Immunosuppressant medications and mortality in inflammatory bowel disease. *Am J Gastroenterol* 2008; **103**: 1428-1435; quiz 1436
4 **Shibolet O**, Karmeli F, Eliakim R, Swennen E, Brigidi P, Gionchetti P, Campieri M, Morgenstern S, Rachmilewitz D. Variable response to probiotics in two models of experimental colitis in rats. *Inflamm Bowel Dis* 2002; **8**: 399-406
5 **Guarner F**, Malagelada JR. Gut flora in health and disease. *Lancet* 2003; **361**: 512-519
6 **Dieleman LA**, Goerres MS, Arends A, Sprengers D, Torrice C, Hoentjen F, Grenther WB, Sartor RB. Lactobacillus GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. *Gut* 2003; **52**: 370-376
7 **Rioux KP**, Fedorak RN. Probiotics in the treatment of inflammatory bowel disease. *J Clin Gastroenterol* 2006; **40**: 260-263
8 **Fedorak RN**, Madsen KL. Probiotics and the management of inflammatory bowel disease. *Inflamm Bowel Dis* 2004; **10**: 286-299
9 **Bibiloni R**, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, De Simone C, Sartor RB. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. *Am J Gastroenterol* 2005; **100**: 1539-1546
10 **Kruis W**, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, Kamm MA, Weismueller J, Beglinger C, Stolte M, Wolff C, Schulze J. Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. *Gut* 2004; **53**: 1617-1623
11 **Kokesova A**, Frolova L, Kverka M, Sokol D, Rossmann P, Bartova J, Tlaskalova-Hogenova H. Oral administration of probiotic bacteria (E. coli Nissle, E. coli O83, Lactobacillus casei) influences the severity of dextran sodium sulfate-induced colitis in BALB/c mice. *Folia Microbiol* (Praha) 2006; **51**: 478-484
12 **Peran L**, Camuesco D, Comalada M, Bailon E, Henriksson A, Xaus J, Zarzuelo A, Galvez J. A comparative study of the preventative effects exerted by three probiotics, Bifidobacterium lactis, Lactobacillus casei and Lactobacillus acidophilus, in the TNBS model of rat colitis. *J Appl Microbiol*

2007; **103**: 836-844
13 **Chapman TM**, Plosker GL, Figgitt DP. Spotlight on VSL#3 probiotic mixture in chronic inflammatory bowel diseases. *BioDrugs* 2007; **21**: 61-63
14 **Chapman TM**, Plosker GL, Figgitt DP. VSL#3 probiotic mixture: a review of its use in chronic inflammatory bowel diseases. *Drugs* 2006; **66**: 1371-1387
15 **Schultz M**. Clinical use of E. coli Nissle 1917 in inflammatory bowel disease. *Inflamm Bowel Dis* 2008; **14**: 1012-1018
16 **Hamamoto N**, Maemura K, Hirata I, Murano M, Sasaki S, Katsu K. Inhibition of dextran sulphate sodium (DSS)-induced colitis in mice by intracolonically administered antibodies against adhesion molecules (endothelial leucocyte adhesion molecule-1 (ELAM-1) or intercellular adhesion molecule-1 (ICAM-1)). *Clin Exp Immunol* 1999; **117**: 462-468
17 **Fabia R**, Ar'Rajab A, Johansson ML, Willen R, Andersson R, Molin G, Bengmark S. The effect of exogenous administration of Lactobacillus reuteri R2LC and oat fiber on acetic acid-induced colitis in the rat. *Scand J Gastroenterol* 1993; **28**: 155-162
18 **Dieleman LA**, Palmen MJ, Akol H, Bloemena E, Pena AS, Meuwissen SG, Van Rees EP. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* 1998; **114**: 385-391
19 **Torres MI**, Rios A. Current view of the immunopathogenesis in inflammatory bowel disease and its implications for therapy. *World J Gastroenterol* 2008; **14**: 1972-1980
20 **Hudcovic T**, Stepankova R, Kozakova H, Hrncir T, Tlaskalova-Hogenova H. Effects of monocolonization with Escherichia coli strains O6K13 and Nissle 1917 on the development of experimentally induced acute and chronic intestinal inflammation in germ-free immunocompetent and immunodeficient mice. *Folia Microbiol* (Praha) 2007; **52**: 618-626
21 **Kamada N**, Maeda K, Inoue N, Hisamatsu T, Okamoto S, Hong KS, Yamada T, Watanabe N, Tsuchimoto K, Ogata H, Hibi T. Nonpathogenic Escherichia coli strain Nissle 1917 inhibits signal transduction in intestinal epithelial cells. *Infect Immun* 2008; **76**: 214-220
22 **Balish E**, Warner T. Enterococcus faecalis induces inflammatory bowel disease in interleukin-10 knockout mice. *Am J Pathol* 2002; **160**: 2253-2257
23 **Ruiz PA**, Shkoda A, Kim SC, Sartor RB, Haller D. IL-10 gene-deficient mice lack TGF-beta/Smad signaling and fail to inhibit proinflammatory gene expression in intestinal epithelial cells after the colonization with colitogenic Enterococcus faecalis. *J Immunol* 2005; **174**: 2990-2999
24 **Ashwood P**, Harvey R, Verjee T, Wolstencroft R, Thompson RP, Powell JJ. Functional interactions between mucosal IL-1, IL-ra and TGF-beta 1 in ulcerative colitis. *Inflamm Res* 2004; **53**: 53-59
25 **Carol M**, Lambrechts A, Van Gossum A, Libin M, Goldman M, Mascart-Lemone F. Spontaneous secretion of interferon gamma and interleukin 4 by human intraepithelial and lamina propria gut lymphocytes. *Gut* 1998; **42**: 643-649
26 **Kucharzik T**, Lugering N, Weigelt H, Adolf M, Domschke W, Stoll R. Immunoregulatory properties of IL-13 in patients with inflammatory bowel disease; comparison with IL-4 and IL-10. *Clin Exp Immunol* 1996; **104**: 483-490

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BRIEF ARTICLES

Evolution and predictive factors of thyroid disorder due to interferon alpha in the treatment of hepatitis C

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Abstract

AIM: To study predictive factors of thyroid dysfunction associated with interferon-alpha (IFNα) therapy in chronic hepatitis C (CHC) and to describe its long-term evolution in a large population without previous thyroid dysfunction.

METHODS: We performed a follow-up of thyroid function and detection of thyroid antibodies in 301 patients treated for CHC with IFNα from 1999 to 2004.

RESULTS: Thyroid disorder developed in 30/301 (10%) patients with a mean delay of 6 ± 3.75 mo: 13 patients had hyperthyroidism, 11 had hypothyroidism, and 6 had biphasic evolution. During a mean follow-up of 41.59 ± 15.39 mo, 9 patients with hyperthyroidism, 3 with hypothyroidism, and 4 with biphasic evolution normalized thyroid function in 7.88 ± 5.46 mo. Recovery rate of dysthyroidism was not modified by treatment discontinuation, but was better for patients with negative thyroid antibodies before antiviral treatment (P = 0.02). Women had significantly more dysthyroidism (P = 0.05). Positive thyroid peroxidase and thyroglobulin antibodies were more frequent before antiviral treatment in patients who developed dysthyroidism (P < 0.0003 and P = 0.0003, respectively). In a multivariate model, low fibrosis was found to be a predictive factor of dysthyroidism (P = 0.039).

CONCLUSION: In this monocentric population of CHC, dysthyroidism, especially hyperthyroidism, developed in 10% of patients. Low fibrosis was found to be a predictive factor of dysthyroidism. Thyroid disorder recovered in 16/30 patients (53%) and recovery was better in the non-autoimmune form.

Key words: Chronic hepatitis C; Interferon alpha; Predictive factors; Thyroid disorder

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INTRODUCTION

Three alpha interferons and two peg-interferons are currently commercially available for the treatment of chronic hepatitis C (CHC). Since 2001, peg-interferons have been used in association with ribavirin[1,2], and they have become the reference treatment for CHC since the French consensus conference in 2002[3]. In the presence or absence of ribavirin, interferon-alpha (IFNα) has a well-known side effect profile. Some side effects are common, such as pseudo-flu syndrome, headaches, myalgia, fever, wasting, leucopoenia or thrombocytopoenia. Indeed, clinicians often reduce the dose or sometimes discontinue IFNα in those patients who develop thyroid dysfunction, thus possibly compromising the therapeutic response to this treatment. In 1995, Preziati *et al*[4] reported that 9.3% of patients with CHC receiving IFNα developed thyroid dysfunction. During the treatment of CHC, IFNα-induced thyroid dysfunction appears in 3% to 15% of cases[5-9], with various clinical presentations. In previous

studies, the number of patients included was insufficient in certain cases[4,5,10,11], and other studies did not exclude patients with a past history of dysthyroidism[4,8,9,12,13]. However, the long-term course and the risk factors of thyroid disorder are not well understood[6,7,14].

In this single-center study we report on a large population of patients without previous thyroid dysfunction who underwent IFNα treatment for CHC. Our objectives were to describe the prevalence and long-term course of thyroid disorder in this population and to assess the factors that are predictive of dysthyroidism.

MATERIALS AND METHODS

Patients

We studied all patients with CHC, treated with IFNα from January, 1999 to May, 2004 at the Department of Hepatology and Gastroenterology at Bicêtre Hospital (Kremlin-Bicêtre, France). Patients with human immunodeficiency virus or hepatitis B virus co-infection, hemophiliacs and patients with a past history of thyroid disorder were systematically excluded. Before 2002, a liver biopsy was performed in each patient in order to evaluate inflammatory activity and the stage of liver fibrosis measured by the Metavir score[15]. Since 2002, liver biopsy was performed only in patients with genotype 1, 4 or 5. Patients received standard interferon alpha (2a), 3 MU subcutaneously thrice weekly, peg-interferon alpha (2b) (Viraferon peg®, Schering Plough, NJ, USA) 1.5 μg per kilogram of body weight subcutaneously once weekly or peg-interferon alpha (2a) (Pegasys®, Hoffmann-La Roche, Ltd, Switzerland) 180 μg subcutaneously once weekly, with or without 800 mg to 1200 mg of ribavirin per day. Thyroid-stimulating hormone (TSH) was measured before and every eight weeks during the antiviral treatment. Therapeutic follow-up of thyroid disorder was then performed until June, 2006. Thyroid peroxidase antibodies (TPOAb), thyroglobulin antibodies (TgAb) and thyroid-stimulating hormone receptor antibodies (TSHRAb) were measured before the start of antiviral treatment and after the diagnosis of thyroid disorder as necessary.

Methods

The diagnosis of CHC was based on positive hepatitis C virus antibodies assessed by a second or third generation enzyme immunoassay. Hepatitis C virus RNA was measured by polymerase chain reaction amplification of viral RNA from serum. Viral genotypes were determined using a hybridization technique (INNO-LIPA HCV, Innogenetics, Gent, Belgium). The serum levels of TSH were measured using the AutoDELFIA™ TSH Ultra assay (sensitivity 0.03 MIU/L, total analytical variation < 5%) from Wallacoy, Turku, Finland. The normal TSH range in our laboratory was 0.3-4.0 MIU/L. The serum levels of TPOAb, TgAb and TSHRAb were measured using radioimmunoassays or radioimmunometric assays. The normal ranges in our laboratory were TPOAb < 60 IU/L, TgAb < 60 IU/L and TSHRAb < 5 IU/L.

Thyroid dysfunction was diagnosed when TSH level was either < 0.3 MIU/L (hyperthyroidism) or > 4.0 MIU/L (hypothyroidism) by two successive tests. Thyroid ultrasonography or thyroid scintigraphy was performed according to the clinical judgement of the endocrinologist. We have previously found three profiles of dysthyroidism: hyperthyroidism, hypothyroidism and a short hyper-followed by long hypothyroidism classically named biphasic evolution[9,14,16].

Table 1 Characteristics of the study population

Characteristics	Total group (n = 301)
Age[1] (yr)	48.27 ± 11.79
Gender (%, M/F)	57.5/42.5
Contamination mode (%)	
Tranfusion	27.2
Drug-addict	26.9
Blood exposure accident	7.6
Others	38.2
Genotype (% with 1/2/3/4/5/ND)	35/11/18/8/2/26
Stage of fibrosis (% with stage 0/1/2/3/4/ND)	1/11/42/20/20/6
Type of treatment (%)	
With IFNα	3.3
Peg-interferon	9.0
IFNα + ribavirin	27.2
Peg-interferon + ribavirin	60.4
Duration of treatment (mo)[1]	7.91 ± 3.78
TSH before treatment (MIU/L)[1]	1.54 ± 1.25
Positive antibodies before treatment	
TPOAb	12/229
TGAb	8/227
TSHRAb	1/95

ND: Not determined. [1]mean ± SD.

Statistical analysis

The predictive values of the following factors were analyzed: patients age at onset of the antiviral treatment, gender, mode of contamination, viral load and genotype, grade of histological fibrosis, type and duration of the antiviral therapy, TSH levels and the presence of TgAb, TPOAb or TSHRAb before the antiviral treatment.

Descriptive statistics were obtained using the Kruskal-Wallis test as appropriate, followed by a multivariate logistic regression analysis. A two-tailed P value < 0.05 was considered significant. Data analysis was performed using the EPI-Info Statistical Package (version 3.2.2).

RESULTS

Characteristics of the study population

The main characteristics of the 301 studied patients are shown in Table 1. Genotype status was known in 224 (74%) of 301 patients, as it was not known for the patients treated before 2002. The stage of fibrosis based on the Metavir score was obtained for 94% of patients. Patients with genotype 2 or 3 treated after 2002 did not have systematic evaluation of fibrosis before antiviral treatment. 247 (87%) of the patients who had a biopsy, had moderate or severe fibrosis (equal to or more than F2). In the inclusion period, from 1999 to 2004, there was heterogeneity in the antiviral treatment. However, the majority (60.4%) of the study population received

Table 2 Classification of thyroid disorder and long-term normalisation of dysthyroidism (n = 30)

	Type of dysthyroidism of normalisation	Discontinued treatment	Normalisation	Mean delay (mo)
Hyperthyroidism	13	9	9	7.44 ± 7.05[1]
Silent thyroiditis	5	4	5	6.80 ± 3.70[1]
Graves' disease	4	2	1	5
NC	2	2	2	2.00 ± 1.41[1]
Triphasic evolution	2	1	1	24
Hypothyroidism	11	6	3	10.67 ± 4.04[1]
Autoimmune	10	5	2	8.50 ± 2.12[1]
NC	1	1	1	15
Biphasic evolution	6	3	4	10.75 ± 5.50[1]

NC: Not classified. [1]mean ± SD.

peg-interferon alpha and ribavirin bitherapy. The TSH level before the antiviral treatment was known for all studied patients and was within normal ranges.

Prevalence of thyroid dysfunction during the antiviral treatment

Amongst the 301 patients with CHC, 30 (10%) developed biochemical thyroid dysfunction (TSH < 0.3 or > 4.0 MIU/L) during the antiviral treatment, 17 women and 13 men. Hyperthyroidism was seen in 13 (43%) of the 30 cases, hypothyroidism in 11 (37%) and biphasic evolution in 6 (20%). Table 2 shows the prevalence and classification of the thyroid disorders. The investigative work-up performed for each case with thyroid dysfunction, classified 11 of the 13 patients with hyperthyroidism as Graves' disease, silent thyroiditis or triphasic evolution, and 10 of the 11 patients with hypothyroidism as autoimmune hypothyroidism. Graves' disease was defined by the presence of clinical hyperthyroidism with positive TSHRAb and diffusely increased radioactive iodine intake on thyroid scintigraphy. Silent thyroiditis was defined by thyrotoxicosis, no tender goitre, and markedly decreased radioactive iodine intake on thyroid scintigraphy. Cases of "triphasic evolution" were defined as reported by Bohbot *et al* in 2006[17] (unusual evolution of silent thyroiditis to Graves' disease). Autoimmune hypothyroidism was defined by positive TgAb and/or TPOAb associated with clinical hypothyroidism.

Long-term course of dysthyroidism

Dysthyroidism occurred at an average of 6 ± 3.75 mo after the beginning of antiviral treatment. The evolution of the different profiles of dysthyroidism was described (Table 2) during a long-term follow-up (41.59 ± 15.35 mo) after the diagnosis of dysthyroidism. We observed that practitioners did not have the same attitude with respect to the evolution of dysthyroidism because the antiviral treatment was more frequently discontinued in hyperthyroidism (69%) than in hypothyroidism (55%) or biphasic evolution (50%). Four patients with hyperthyroidism did not require discontinuation of antiviral treatment. Among them, 1 patient presented with transient Graves' disease and another died before the end of follow up, 1 patient continued the antiviral treatment for only one month because hyperthyroidism occurred at the end of antiviral treatment and the type of hyperthyroidism could not be classified in 1 patient. Hypothyroidism that needed discontinuation of antiviral treatment was more frequently autoimmune hypothyroidism with TSH > 50 MIU/L except in 3 patients (1 with not classified with hypothyroidism and 2 with moderate elevation of TSH). Concerning therapeutic normalisation, no difference was observed regarding the discontinuation of antiviral treatment (56.6% *vs* 50.0%, NS). Treatment for thyroid disease was administered to 14 symptomatic patients (5 patients received carbimazole and 9 levothyroxine).

Prevalence of positive thyroid antibodies before antiviral treatment

Amongst the patients tested for TPOAb (n = 229), TgAb (n = 227) and TSHRAb (n = 94) before antiviral treatment, 12 (5%) were found to be positive for TPOAb (> 60 IU/L), 8 (3%) positive for TgAb (> 60 IU/L), and only 1 (1%) for TSHRAb (>5 IU/L). None of these patients had thyroid disorder before the introduction of IFNα. 7/12 patients with positive TPOAb and 4/8 patients with positive TgAb developed thyroid disorder during the antiviral treatment. The patients who had positive pretherapeutic TSHRAb did not develop Graves' disease. Regarding the presence of autoantibodies, IFNα induced-thyroid disease was classified as "autoimmune form" and "non-autoimmune form" similar to Mandac *et al*[18]. The autoimmune form was defined by the development of thyroid antibodies with or without clinical disease, including both autoimmune hypothyroidism and Graves' disease. The non-autoimmune form was defined by destructive thyroiditis or hypothyroidism with negative thyroid antibodies. We observed that patient recovery was significantly better in the non-autoimmune form than in the autoimmune form (33.3% *vs* 66.7%, P = 0.02).

Prediction of thyroid dysfunction

As shown in Table 3, we initially performed a univariate analysis using eight covariates (age, gender, contamination mode, genotype, stage of histological fibrosis, type of antiviral treatment [monotherapy with standard IFNα or peg-interferon versus combination of standard IFNα or peg-interferon with ribavirin] and duration, positive autoantibodies before the antiviral

Table 3 Features associated with dysthyroidism

	With dysthyroidism	Without dysthyroidism	*P*
Age[1] (yr)	46.20 ± 10.08	48.49 ± 11.96	0.40
Female (%)	56.9	40.9	0.05
Contamination mode (%)			0.33
Transfusion	33.3	26.6	
Drug-addict	20.0	27.7	
Blood exposure accident	13.3	7.0	
Others	33.3	38.7	
Genotype (%, 1/2/3/4/5/6/ND)	57/13/13/0/0/0/17	33/11/20/9/1/0/26	0.20
Stage of fibrosis < F2 (%)	30.0	10.3	0.009
Type of treatment (%)		0.47	
IFNα	3.3	3.3	
Peg-interferon	6.7	9.3	
IFNα + ribavirin	16.7	28.5	
Peg-interferon + ribavirin	73.3	58.9	
Duration of treatment[1] (mo)	7.73 ± 3.64	8.09 ± 3.93	0.30
Positive antibodies before treatment (%)			
TPOAb	26.9	2.5	< 0.0003
TgAb	18.5	1.5	0.0003
TSHRAb	0.0	1.3	0.42

ND: Not determined. [1]mean ± SD.

treatment: TPOAb, TgAb and TSHRAb). Four covariates were associated with dysthyroidism (gender, stage of histological fibrosis, positive TPOAb and TgAb). Secondly, in a multivariate logistic regression analysis of predictive factors of dysthyroidism using those four covariates, one predictive factor was found. The index of fibrosis was significantly less for patients with dysthyroidism than for patients without dysthyroidism. The stage of fibrosis was less than 2 units (mild fibrosis) in 30.0% of patients with dysthyroidism *vs* 10.3% of patients without dysthyroidism (OR, 0.56; 95% IC, 0.33-0.97; $P = 0.039$). There was a non significant trend towards positive TPOAb before antiviral treatment for patients with dysthyroidism. Amongst patients with positive TPOAb before antiviral treatment, 7 (26.9%) developed dysthyroidism *vs* 5 (2.5%) who did not (OR, 5.31; 95% IC, 0.80-35.16; $P = 0.083$).

DISCUSSION

The prevalence of thyroid dysfunction during IFNα therapy for CHC was 10% in our series. Amongst the 301 patients with CHC, hyperthyroidism was more frequent (13/30) than hypothyroidism or biphasic evolution. The mean follow-up of thyroid disorder in our study was 41.59 ± 15.39 mo, 53% of patients recovered from thyroid disease without a difference regarding the discontinuation of antiviral treatment. Mild fibrosis was found to be an independent predictive factor of dysthyroidism during antiviral treatment.

Our single center study included a large population of 301 patients with CHC and we performed a long term follow-up of these patients, not only during the antiviral treatment, but also after treatment, to detect dysthyroidism in patients who had no previous thyroid dysfunction. Although the patient data were retrospective, the follow-up data were partly prospective. This may explain some of the heterogeneity in the type of antiviral treatment used. In addition, the conditions under which the antiviral treatment was stopped when dysthyroidism developed were not well defined. We evaluated the presence of positive thyroid antibodies using the same methods in all patients, and an investigative work-up of the pathology was performed for each case of thyroid dysfunction.

The prevalence of hyperthyroidism found in our study (43% *vs* 37% hypothyroidism) is unusual. Previous studies have reported more hypothyroidism (two out of the three cases) than hyperthyroidism (one out of the three cases)[19] with the exception of Benelhadj *et al*[5] and Hsieh *et al*[11]. Hsieh *et al*[11] explained this difference as being related to the population's eating habits, yet our study population was not particularly exposed to an increased risk of dysthyroidism due to eating habits. Benelhadj *et al*[5] did not explain this difference as only 6 patients developed thyroid dysfunction. The discrepancy may be partly explained by the findings of several other studies including silent thyroiditis developing into hypothyroidism or biphasic evolution whereas this disease usually begins with hyperthyroidism. Furthermore, in our series, hyperthyroidism cases included 30% (4/13) with Graves' disease, which is in the same range as a previously published series[20].

In accordance with the presence of at least one thyroid antibody, we classified thyroid disorder, as autoimmune and non-autoimmune, which seemed to be predictive of the evolution of dysthyroidism. In this study we should have based the autoimmune form on at least one positive thyroid antibody rather than consider each positive antibody separately. Three of the four cases of Graves' disease developed following IFNα therapy and did not recover after the end of the antiviral therapy. This suggests that IFNα triggered the development of Graves' disease in predisposed individuals[20]. In silent thyroiditis, which is a non-autoimmune IFNα-induced thyroiditis, four patients recovered without

the addition of specific treatment when interferon was discontinued and one recovered without discontinuing antiviral treatment. This suggests that the autoimmune mechanism is more deleterious in IFNα-induced thyroid disease. Among the eleven hypothyroidism patients, therapeutic normalisation was obtained in 3 (27%) within 10.67 ± 4.04 mo. Also, the patients who developed autoimmune forms of hypothyroidism, such as autoimmune hypothyroidism, did not recover after cessation of IFNα treatment and systematically needed T4 replacement during the follow-up.

In the multivariate analysis, one factor was significantly correlated with the development of dysthyroidism during antiviral treatment: the stage of fibrosis below the F2 Metavir score. However, patients treated with IFNα had more severe fibrosis (82% of patients with a stage of fibrosis equal to or above F2). Perhaps this was correlated to the variability in the autoimmune response to hepatitis C virus infection, however, this predictive factor will require further study. Surprisingly, the presence of TPOAb before the introduction of antiviral treatment was not significant in the multivariate model whereas it was in the univariate analysis; this may have been due to the small number of patients with positive antibodies. Kabbaj *et al*[21], found three predictive factors for dysthyroidism in a univariate analysis: female gender, positive anti TPO antibodies before antiviral treatment and TSH before antiviral treatment (even if it was still in the normal ranges). We do not understand why the variable "stage of fibrosis under F2" is mentioned in the statistical analysis because only patients with fibrosis equal or more than F2 were treated. Kee *et al*[22], found that only female gender was predictive of dysthyroidism in a multivariate model. Thyroid microsomal antibody was found to be predictive of thyroid disease in a case-control study. There were no significant differences between thyroid dysfunction patients in the case-control study with respect to liver inflammation and fibrosis grade, however, the authors used the Knodell score which does not distinguish activity and fibrosis.

Some practical guidelines may be drawn from this study: the TPOAb state should be determined in patients before introducing IFNα and a regular follow-up of TSH every two mo or less is needed in patients with a risk of dysthyroidism (low fibrosis, female gender, positive TPOAb). Finally, two distinct mechanisms are described in the development of thyroid disorder during IFNα therapy: autoimmune and non-autoimmune-induced thyroid dysfunction. With regard to our results, the autoimmune form seems to have more severe consequences and longer evolution, which indicates the importance of early detection, in order to adapt the follow-up of thyroid function and therapy without discontinuing the antiviral treatment, since the discontinuation of antiviral treatment seems to have no predictive value on the evolution of dysthyroidism.

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COMMENTS

Background

Alpha interferons and peg-interferons have successively become the reference treatment for chronic hepatitis C with or without ribavirin. They have both induced thyroid dysfunction in 3% to 15% of cases. Indeed, clinicians often reduce the dose or sometimes discontinue interferon-alpha (IFNα) in patients who develop thyroid dysfunction, thus possibly compromising the therapeutic response to this treatment. In previous studies, the number of patients included was insufficient in certain cases, and other studies did not exclude patients with a past history of dysthyroidism. However, the long-term course and the risk factors for thyroid disorder are not well understood.

Research frontiers

Despite the role of IFNα, the pathogenesis of thyroid disease remains uncertain; also it seems to be related to an immunologic predisposition. Therefore, the authors tried to determine the risk factors which influence thyroid dysfunction.

Innovations and breakthroughs

Two distinct mechanisms are described for the development of thyroid disorder during IFNα therapy: autoimmune and non-autoimmune- induced thyroid dysfunction. With regard to our results, the autoimmune form seems to have more severe consequences and longer evolution, which indicates the importance of early detection, in order to adapt the follow-up of thyroid function and therapy without discontinuing the antiviral treatment, since the discontinuation of antiviral treatment seems to have no predictive value on the evolution of dysthyroidism. Furthermore, the stage of fibrosis below the F2 Metavir score was significantly correlated with the development of dysthyroidism during antiviral treatment. We hypothesized that low fibrosis, associated with better HCV response, was also associated with autoimmune activation, including the development of anti-thyroid autoantibodies.

Applications

Some practical guidelines may be drawn from this study: the TPOAb state in patients should be determined before introducing IFNα and a regular follow-up of TSH every two mo or less is needed in patients with a risk of dysthyroidism (low fibrosis, female gender, positive TPOAb).

Peer review

This is a fairly good written manuscript. But the authors need to deal with the issue of predictive factors for developing dysthyroidism in detail in the discussion section and make a plausible explanation about the difference from the previous papers.

REFERENCES

1 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

2 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982

3 **Consensus conference**. Treatment of hepatitis C. *Gastroenterol Clin Biol* 2002; **26** Spec No 2: B303-B320

4 **Preziati D**, La Rosa L, Covini G, Marcelli R, Rescalli S, Persani L, Del Ninno E, Meroni PL, Colombo M, Beck-Peccoz P. Autoimmunity and thyroid function in patients with chronic active hepatitis treated with recombinant interferon alpha-2a. *Eur J Endocrinol* 1995; **132**: 587-593

5 **Benelhadj S**, Marcellin P, Castelnau C, Colas-Linhart N, Benhamou JP, Erlinger S, Bok B. Incidence of dysthyroidism during interferon therapy in chronic hepatitis C. *Horm Res*

1997; **48**: 209-214
6 **Carella C**, Mazziotti G, Morisco F, Manganella G, Rotondi M, Tuccillo C, Sorvillo F, Caporaso N, Amato G. Long-term outcome of interferon-alpha-induced thyroid autoimmunity and prognostic influence of thyroid autoantibody pattern at the end of treatment. *J Clin Endocrinol Metab* 2001; **86**: 1925-1929
7 **Dalgard O**, Bjoro K, Hellum K, Myrvang B, Bjoro T, Haug E, Bell H. Thyroid dysfunction during treatment of chronic hepatitis C with interferon alpha: no association with either interferon dosage or efficacy of therapy. *J Intern Med* 2002; **251**: 400-406
8 **Marazuela M**, Garcia-Buey L, Gonzalez-Fernandez B, Garcia-Monzon C, Arranz A, Borque MJ, Moreno-Otero R. Thyroid autoimmune disorders in patients with chronic hepatitis C before and during interferon-alpha therapy. *Clin Endocrinol* (Oxf) 1996; **44**: 635-642
9 **Mekkakia-Benhabib C**, Marcellin P, Colas-Linhart N, Castel-Nau C, Buyck D, Erlinger S, Bok B. [Natural history of dysthyroidism during interferon treatment of chronic hepatitis C] *Ann Endocrinol* (Paris) 1996; **57**: 419-427
10 **Roti E**, Minelli R, Giuberti T, Marchelli S, Schianchi C, Gardini E, Salvi M, Fiaccadori F, Ugolotti G, Neri TM, Braverman LE. Multiple changes in thyroid function in patients with chronic active HCV hepatitis treated with recombinant interferon-alpha. *Am J Med* 1996; **101**: 482-487
11 **Hsieh MC**, Yu ML, Chuang WL, Shin SJ, Dai CY, Chen SC, Lin ZY, Hsieh MY, Liu JF, Wang LY, Chang WY. Virologic factors related to interferon-alpha-induced thyroid dysfunction in patients with chronic hepatitis C. *Eur J Endocrinol* 2000; **142**: 431-437
12 **Fernandez-Soto L**, Gonzalez A, Escobar-Jimenez F, Vazquez R, Ocete E, Olea N, Salmeron J. Increased risk of autoimmune thyroid disease in hepatitis C vs hepatitis B before, during, and after discontinuing interferon therapy. *Arch Intern Med* 1998; **158**: 1445-1448
13 **Deutsch M**, Dourakis S, Manesis EK, Gioustozi A, Hess G, Horsch A, Hadziyannis S. Thyroid abnormalities in chronic viral hepatitis and their relationship to interferon alfa therapy. *Hepatology* 1997; **26**: 206-210
14 **Moncoucy X**, Leymarie F, Delemer B, Levy S, Bernard-Chabert B, Bouche O, Jolly D, Diebold MD, Cadiot G, Thiefin G. Risk factors and long-term course of thyroid dysfunction during antiviral treatments in 221 patients with chronic hepatitis C. *Gastroenterol Clin Biol* 2005; **29**: 339-345
15 **Bedossa P**, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289-293
16 **Chedin P**, Chanson P, Duranteau L, Guillausseau PJ, Lubetzki J. [Dysthyroidism in patients treated with interferon alpha] *Presse Med* 1994; **23**: 1659-1663
17 **Bohbot NL**, Young J, Orgiazzi J, Buffet C, Francois M, Bernard-Chabert B, Lukas-Croisier C, Delemer B. Interferon-alpha-induced hyperthyroidism: a three-stage evolution from silent thyroiditis towards Graves' disease. *Eur J Endocrinol* 2006; **154**: 367-372
18 **Mandac JC**, Chaudhry S, Sherman KE, Tomer Y. The clinical and physiological spectrum of interferon-alpha induced thyroiditis: toward a new classification. *Hepatology* 2006; **43**: 661-672
19 **Broussolle C**, Steineur MP, Bailly F, Zoulim F, Trepo C. [Hepatitis C virus infection and thyroid diseases] *Rev Med Interne* 1999; **20**: 766-773
20 **Carella C**, Mazziotti G, Amato G, Braverman LE, Roti E. Clinical review 169: Interferon-alpha-related thyroid disease: pathophysiological, epidemiological, and clinical aspects. *J Clin Endocrinol Metab* 2004; **89**: 3656-3661
21 **Kabbaj N**, Guedira MM, El Atmani H, El Alaoui M, Mohammadi M, Benabed K, Lachkar H, Benaissa A. Thyroid disorders during interferon alpha therapy in 625 patients with chronic hepatitis C: a prospective cohort study. *Ann Endocrinol* (Paris) 2006; **67**: 343-347
22 **Kee KM**, Lee CM, Wang JH, Tung HD, Changchien CS, Lu SN, Wang PW. Thyroid dysfunction in patients with chronic hepatitis C receiving a combined therapy of interferon and ribavirin: incidence, associated factors and prognosis. *J Gastroenterol Hepatol* 2006; **21**: 319-326

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BRIEF ARTICLES

Prevalence of bile reflux in gastroesophageal reflux disease patients not responsive to proton pump inhibitors

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Abstract

AIM: To determine the prevalence and characteristics of bile reflux in gastroesophageal reflux disease (GERD) patients with persistent symptoms who are non-responsive to medical therapy.

METHODS: Sixty-five patients (40 male, 25 female; mean age, 50 ± 7.8 years) who continued to report symptoms after 8 wk of high-dose proton pump inhibitor (PPI) therapy, as well as 18 patients with Barrett's esophagus, were studied. All patients filled out symptom questionnaires and underwent endoscopy, manometry and combined pH-metry and bilimetry.

RESULTS: There were 4 groups of patients: 22 (26.5%) without esophagitis, 24 (28.9%) grade A-B esophagitis, 19 (22.8%) grade C-D and 18 (21.6%) Barrett's esophagus. Heartburn was present in 71 patients (85.5%) and regurgitation in 55 (66.2%), with 44 (53%) reporting simultaneous heartburn and regurgitation. The prevalence of pathologic acid reflux in the groups without esophagitis and with grades A-B and C-D esophagitis was 45.4%, 66.6% and 73.6%, respectively. The prevalence of pathologic bilirubin exposure in these 3 groups was 53.3%, 75% and 78.9%, respectively. The overall prevalence of bile reflux in non-responsive patients was 68.7%. Pathologic acid and bile reflux was observed in 22.7% and 58.1% of non-esophagitic patients and esophagitic patients, respectively.

CONCLUSION: The high percentage of patients poorly responsive to PPI therapy may result from poor control of duodenogastroesophageal reflux. Many patients without esophagitis have simultaneous acid and bile reflux, which increases with increasing esophagitis grade.

Key words: Gastroesophageal reflux disease; Duodenogastric reflux; Bile reflux; Bilirubin; Barrett's esophagus

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INTRODUCTION

As a result of their strong acid suppression, proton pump inhibitors (PPIs) have been used to treat most patients with gastroesophageal reflux disease (GERD)[1-5]. Acid reflux is the main risk factor for GERD, with pH-metry being the standard method used in the diagnosis of GERD. Many patients with typical GERD symptoms, however, have been found to have a negative pH-metry[6]; these patients have been found to differ in symptoms, response to medical therapy, and endoscopy results from patients with positive pH-metry.

Although the role of acid reflux in GERD has been established, and links between acid and bile reflux have been found, less is known about the role of bile in the pathogenesis of esophageal mucosal damage. Thus, the incidence of GERD, its clinical impact, etiology, evolution and therapeutic implications cannot be determined directly. This limitation, however, was improved by the introduction of bilimetry in clinical practice[7]. This method uses spectrophotometric analysis to measure the

presence of bilirubin in the refluxate, thus providing a direct and reliable measurement of bile reflux.

The combination of pH-metry and bilimetry has increased the sensitivity and accuracy of GERD diagnosis and has shown that increased bile reflux is correlated with increased severity of esophagitis[8-9]. Moreover, other authors showed that a high percentage of GERD patients, poorly responsive to PPI therapy, had mixed acid and bile reflux, or isolated bile reflux[10]. To expand these investigations, we evaluated the prevalence and characteristics of bile reflux in GERD patients with persistent symptoms who were non-responsive to medical therapy.

MATERIALS AND METHODS

Patients

Of 230 patients with heartburn and regurgitation evaluated between January, 2002 and July, 2006, 65 (40 male, 25 female; mean age, 50 ± 7.8 years) continued to report symptoms after 8 wk of high-dose PPI therapy (40 mg esomeprazole *bid*). In addition, 18 patients with Barrett's esophagus were included. All patients were administered symptom questionnaires and underwent endoscopy, perfused esophageal manometry and combined 24-h esophago-gastric pH-bilimetry.

Endoscopy (EGDS)

The presence of esophagitis was classified according to the Los Angeles Classification[11]. The presence of hiatal hernia was determined and esophageal biopsies were used to diagnose Barrett's esophagus.

Perfused esophageal manometry

Manometric evaluation was made without sedation after 1 wk of pharmacologic wash-out and an overnight fast. An 8-channel manometric device (Menfis Biomedical Inc. Bologne, Italy) connected to a low compliance hydro-pump (Arndorfer Medical Specialties, Greendale, Wisconsin, USA) was used. The 8 open tip (4 radial and 4 longitudinal) manometric probe was inserted through the nose into the stomach and lower esophageal sphincter (LES) parameters (pressure, length and postdeglutitive relaxation) were evaluated by a rapid and stationary pull-through technique. Esophageal motor activity (amplitude and duration of waves, percentage of peristaltic and simultaneous post-deglutitive sequences) was evaluated with stationary pull-through after 20 wet and dry swallows.

Twenty-four hours esophago-gastric pH-metry

We performed this test after 1 wk of pharmacologic wash-out. We used a two channel portable recorder (Menfis Biomedical Inc., Bologna, Italy) connected to two glass pH-metric probes (Telemedicine srl., Naples Italy), which were introduced through the nose without any sedation, and placed 5 cm above and 10 cm below the upper and the lower edge of the LES, respectively. The percentage of total time of exposure to pH < 4 (normal value < 4.2%) was determined.

Twenty-four hours esophago-gastric bilimetry

Bilimetric evaluation was performed simultaneously with pH-metry. We utilized a portable recorder (BILITEC 2000, Sinectics Medical Inc.) connected to two optic-fiber probes placed 5 cm above and 10 cm below the upper and the lower edge of the LES, respectively. The percentage of total time of esophageal bilirubin absorbance > 0.14 (normal value < 7%) was determined.

Ethics committee approval

The ethics committee of the Second University of Naples approved our study and verbal consent was obtained from the study participants.

Statistical analysis

Values are expressed as mean ± SD. Data were compared using Student's *t*-test, Fischer's exact test, or the Chi-square test wherever appropriate. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Endoscopy

Endoscopic evaluation divided the 83 patients into 4 groups. Group Ⅰ consisted of 22 (26.5%) non-esophagitic patients, Group Ⅱ consisted of 24 patients (28.9%) with grade A-B esophagitis, Group Ⅲ consisted of 19 patients (22.8%) with grade C-D esophagitis, and Group Ⅳ consisted of 18 patients (21.6%) with Barrett's esophagus; of the latter, nine had short segment Barrett's esophagus (SSBE) and nine had long segment Barrett's esophagus (LSBE, Figure 1). Of the 83 patients, 61 (73.4%) had a hiatal hernia.

Symptoms

The analysis of the symptoms questionnaire showed that 71 patients (85.5%) had heartburn, 55 (66.2%) had regurgitation, and 44 (53%) had simultaneous heartburn and regurgitation. Twelve patients (14.4%) reported nocturnal cough and 7 (8.4%) reported chest pains. Analysis of symptom scores showed no significant between group differences. In contrast, symptom history was significantly higher in Group Ⅲ than in Groups Ⅰ and Ⅱ, but not between Barrett's patients (Figure 2).

Manometric data

Hypotonic LES was observed in 8 of 22 (36.3%) Group Ⅰ, 14 of 24 (58.3%) Group Ⅱ and 14/19 (73.7%) Group Ⅲ patients (Group Ⅰ *vs* Group Ⅲ: $P = 0.0279$). Hypotonic LES was also present in 8 of 9 (88.8%) LSBE and 7 of 9 (77.7%) SSBE patients. Mean LES-P in Group Ⅰ (13.91 ± 4.8 mmHg) was significantly higher than in Groups Ⅱ (9.2 ± 2.2 mmHg, $P < 0.001$), and Ⅲ (8.6 ± 2.8 mmHg, $P < 0.001$) and in SSBE (9.2 ± 3.4 mmHg, $P = 0.0056$) and LSBE (7.1 ± 1.6 mmHg, $P < 0.001$) patients.

Five of the 22 (22.7%) patients in Group Ⅰ showed ineffective esophageal motility, increasing to 50% (12/24) in Group Ⅱ and to 73.6% (14/19) Group Ⅲ.

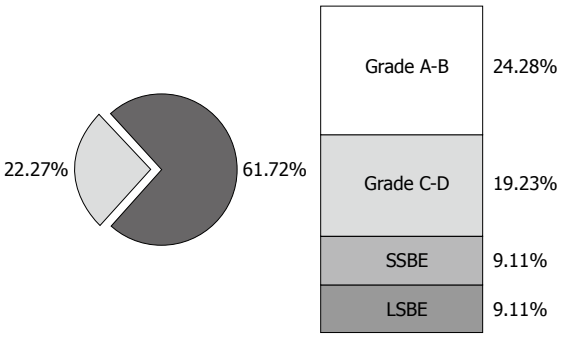

Figure 1 Endoscopy evaluation in 83 gastroesophageal reflux disease (GERD) patients. SSBE: Short segment Barrett's esophagus; LSBE: Long segment Barrett's esophagus.

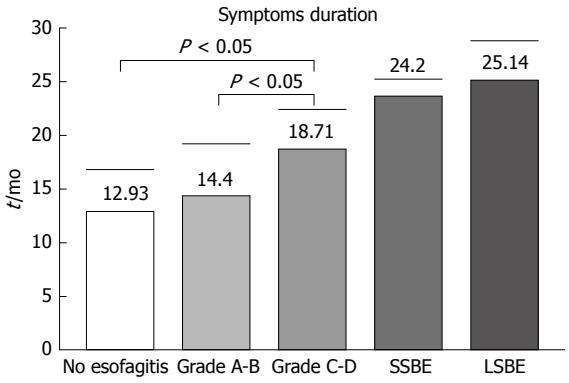

Figure 2 Symptoms. Mean duration of symptoms history in each group of GERD patients (mean ± SD, Fisher's exact test).

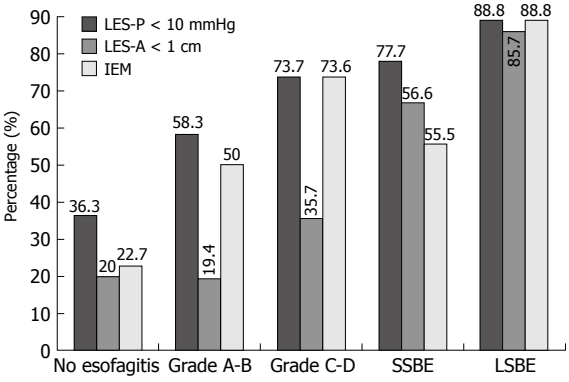

Figure 3 Manometry. Percentage with hypotonic lower esophageal sphincter (LES), Short LES and ineffective esophageal motility in each group of GERD patients (mean ± SD, Fisher's exact test).

In comparison, 5 of 9 SSBE (55.5%) and 8 of 9 (88.8%) LSBE patients showed ineffective esophageal motility (Figure 3).

pH-metric evaluation

The prevalence of pathologic acid reflux increased relative to esophagitis, from 45.4% (10/22) in Group I, to 66.6% (16/24) in Group Ⅱ and 73.6% (14/19) in Group Ⅲ. Six of 9 (66.6%) SSBE and 7 of 9 (77.7%) LSBE patients showed pathologic pH-metry (Figure 4).

Relative time at pH < 4 was 5.1 ± 2.7% in non-esophagitic (Group Ⅰ) patients, increasing to 7.03 ± 3.6% ($P > 0.05$) in Group Ⅱ and 7.2 ± 0.24% ($P > 0.05$) in Group Ⅲ. In contrast, both the SSBE (9.8 ± 5.1%, $P = 0.0022$) and LSBE (15.5 ± 7.7%, $P < 0.0001$) groups had significantly more time at pH < 4 than did Group Ⅰ (Figure 5).

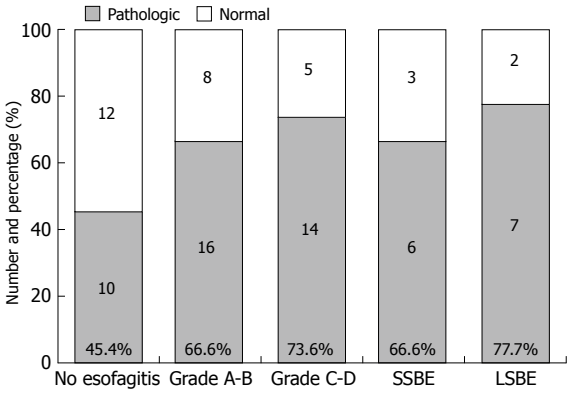

Figure 4 pH-metry. Number and percentage of pathologic and normal pH-metry in each group of GERD patients.

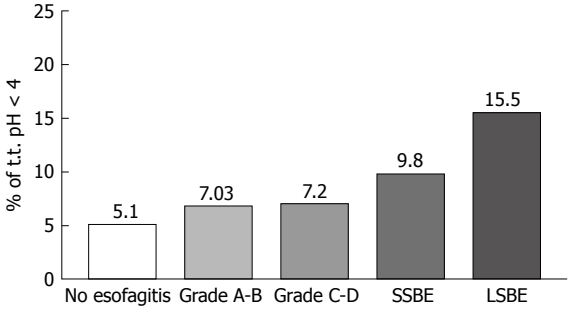

Figure 5 Esophageal acid exposure. Mean value of total time of esophageal exposure at pH < 4 in each group of GERD patients (mean ± SD).

Bilimetric evaluation

Pathologic bilirubin exposure was observed in 9 of 22 (53.3%) Group Ⅰ, 18 of 24 (75%) Group Ⅱ and 15 of 19 (78.9%) Group Ⅲ patients, as well as in 7 of 9 (77.7%) SSBE and 8 of 9 (88.8%) LSBE patients (Figure 6). The global prevalence of patients non-responsive to PPI therapy was 68.7% (57/83).

Mean time of bile absorbance > 0.14 in all patients was 16.9 ± 4.6%, 9.2 ± 5.2% in Group Ⅰ, 10.9 ± 4.6% in Group Ⅱ, 16.3 ± 6.3% in Group Ⅲ, 15.8 ± 6.7% in SSBE and 19.9 ± 6.2% in LSBE (Figure 7).

Combined pH-bilimetry evaluation

The analysis of combined pH-metry and bilimetry showed that 8 of 22 (36.4%) non-esophagitic patients and only 5 of 43 (11.6%) esophagitic patients [3 of 24 (12.5%) in Group Ⅱ and 2 of 19 (10.5%) in Group Ⅲ] had both values within the normal range. None of the 18 Barrett's patients had normal esophageal exposure to acid and bile.

Pathological bilimetry associated with normal pH-metry was observed in 4 of 22 (18.2%) non-esophagitic and 8 of 43 (18.6%) esophagitic patients [5 of 24 (20.8%) in Group Ⅱ and 3 of 19 (15.8%) in Group Ⅲ], as well

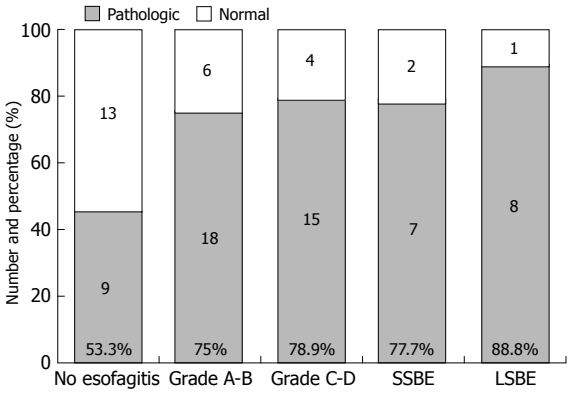

Figure 6 Bilimetry. Number and percentage of pathologic and normal bilimetry in each group of GERD patients.

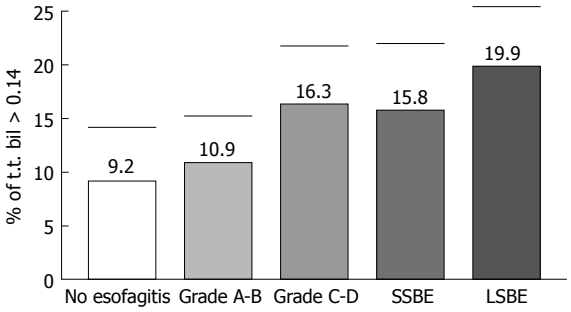

Figure 7 Esophageal bile exposure. Mean value of total time of esophageal bilirubin absorbance > 0.14 in each group of GERD patients (mean ± SD).

as in 3 of 9 (33.3%) SSBE and 2 of 9 (22.2%) LSBE patients.

Conversely, pathological pH-metry associated with normal bilimetry was observed in 5 of 22 (22.7%) non-esophagitic and 5 of 43 (11.6%) esophagitic patients (3 of 24 (12.5%) in Group Ⅱ and 2 of 19 (10/5%) in Group Ⅲ), as well as in 2 of 9 (22.2%) SSBE and 1 of 9 (11.1%) LSBE patients.

Pathologic bilimetry and pathologic pH-metry were observed in 5 of 22 (22.7%) non-esophagitic and 25 of 43 (58.1%) esophagitic patients [13 of 24 (54.2%) in Group Ⅱ and 12 of 19 (66.1%) in Group Ⅲ], as well as in 4 of 9 (44.4%) SSBE and 6 of 9 (66.7%) LSBE patients (Figure 8).

DISCUSSION

Although the introduction of PPIs has improved outcomes in GERD patients, a significant number of patients treated with a high dosage of PPIs (40 mg bid) show no improvements in symptoms or esophagitis. Of patients who do not respond to PPI therapy, however, only 37% show pathological pH-metry results[10]. In contrast, the combination of pH-metry and bilimetry showed pathological results in 70% of patients, thus improving the sensitivity of detection of reflux by 35%. These outcomes are important for the management of GERD patients, in that the constant presence of GERD symptoms, as documented by pH-metry, are probably caused by the incomplete acid-secretion control of the

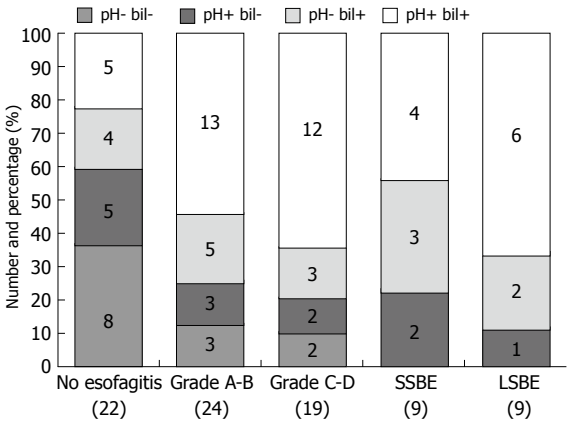

Figure 8 Combined pH-metry and bilimetry evaluation. Number and percentage of various associations of pH-metry and bilimetry in each group of patients.

PPI drugs. The presence of biliary reflux, as documented by bilimetry, suggests that the PPIs are unable to inhibit bile secretion. Persistent symptoms in patients without any documented evidence of acid and/or bile reflux suggests that these patients may be suffering from a less common disease, such as hypersensitive esophagus, or that these symptoms arise from psychiatric causes[12].

In this study, we analyzed patients non-responsive to an 8 wk course of high-dosage PPI therapy. In addition to assessing the presence of both acid and bile reflux, we assessed the features and duration of symptoms and the esophageal motor pattern. Our overall goal was to identify characteristics that could be linked to the persistent symptoms and typical lesions of GERD. We found that a high percentage of patients (36%) were poorly responsive to PPI therapy. When associated with a long symptom history, this characteristic showed a strong correlation with the presence of esophagitis. Compared with patients without esophagitis, those with esophagitis showed a significantly longer history of symptoms, but there was no differences in severity[13-14].

Functionally, manometric analysis has shown that hypotonic and short lower esophageal sphincter was correlated with esophagitis, with hypotonic and short LES having a strong influence on the natural history of GERD[15-16]. We found that these manometric alterations were present in only 36% of patients without esophagitis, increasing to 60% in patients with grade A-B esophagitis and to 70% in patients with grade C-D esophagitis. Moreover, in agreement with findings showing that effective esophageal motility (non peristaltic sequences and waves with amplitude < 15 mmHg) is important[16-17], we found that ineffective esophageal motility, while infrequent in non-esophagitic patients (22%), increased to 50% in patients with grade A-B esophagitis and to 73% in patients with grade C-D esophagitis.

Our findings also showed that a high percentage of GERD patients poorly responsive to PPIs have biliary reflux. We found that a high percentage (53.3%) off non-esophagitic GERD patients had pathologic bile reflux, increasing to 70% in esophagitic patients. In

addition, the percentage of total time of bile absorbance > 0.14 was associated with esophagitis severity.

It is important to emphasize that patients with severe GERD (i.e. presence of esophagitis and/or Barrett's esophagus) showed a significant increase in simultaneous bile and acid reflux relative to that in non-esophagitic GERD patients. Thus, in these patients, the esophageal mucosa is simultaneously exposed to the harmful effects of gastric and duodenal juice, with increased damage correlated with increased exposure. Similarly, animal models have shown that simultaneous exposure of the esophageal mucosa to both acid and bile reflux results in greater mucosal damage than exposure to isolated acid or bile reflux[17]. Moreover, while taurocholate does not cause mucosal damage at neutral pH, it does so at acid pH, as evidenced by ionic permeability studies[18].

On the contrary, the presence of a pathologic bile test without pathologic acid reflux, which was quite common in non-esophagitic patients and those with Grade A-B esophagitis, was observed in only 30% of patients with grade C-D esophagitis. This shows how the evolution of GERD to a more severe grade is influenced not only by acid reflux, but also by the association of acid reflux with duodenogastroesophageal reflux disease.

COMMENTS

Background

The available literature suggests that proton pump inhibitors (PPIs) are less efficacious in normalizing duodeno gastroesophageal reflux disease (DGERD), compared with their effect on acid reflux, in contrast to reflux surgery that has shown to adequately suppress both esophageal acid and bile exposure.

Research frontiers

This study clearly shows that the high percentage of patients poorly responsive to PPI therapy may result from poor control of DGERD. Many patients without esophagitis have simultaneous acid and bile reflux, which increases with increasing esophagitis grade.

Applications

Laparoscopic anti-reflux surgery seems to be the treatment of choice, being effective in suppressing both acid and bilirubin exposure.

Peer review

In this manuscript, the authors ascertained that many PPI-resistant GERD patients have simultaneous acid and bile reflux, which increases with increasing esophagitis grade. The study was well performed and the conclusion was clear.

REFERENCES

1 **Tytgat G**. Long-term GERD management: the individualized approach. *Drugs Today* (Barc) 2006; **42** Suppl B: 23-29

2 **van Pinxteren B**, Numans ME, Bonis PA, Lau J. Short-term treatment with proton pump inhibitors, H2-receptor antagonists and prokinetics for gastro-oesophageal reflux disease-like symptoms and endoscopy negative reflux disease. *Cochrane Database Syst Rev* 2006; **3**: CD002095

3 **Orel R**, Brecelj J, Homan M, Heuschkel R. Treatment of oesophageal bile reflux in children: the results of a prospective study with omeprazole. *J Pediatr Gastroenterol Nutr* 2006; **42**: 376-383

4 **Sarela AI**, Hick DG, Verbeke CS, Casey JF, Guillou PJ, Clark GW. Persistent acid and bile reflux in asymptomatic patients with Barrett esophagus receiving proton pump inhibitor therapy. *Arch Surg* 2004; **139**: 547-551

5 **Netzer P**, Gut A, Brundler R, Gaia C, Halter F, Inauen W. Influence of pantoprazole on oesophageal motility, and bile and acid reflux in patients with oesophagitis. *Aliment Pharmacol Ther* 2001; **15**: 1375-1384

6 **Patti MG**, Diener U, Tamburini A, Molena D, Way LW. Role of esophageal function tests in diagnosis of gastroesophageal reflux disease. *Dig Dis Sci* 2001; **46**: 597-602

7 **Bechi P**, Pucciani F, Baldini F, Cosi F, Falciai R, Mazzanti R, Castagnoli A, Passeri A, Boscherini S. Long-term ambulatory enterogastric reflux monitoring. Validation of a new fiberoptic technique. *Dig Dis Sci* 1993; **38**: 1297-1306

8 **Felix VN**, Viebig RG. Simultaneous bilimetry and pHmetry in GERD and Barrett's patients. *Hepatogastroenterology* 2005; **52**: 1452-1455

9 **Osugi H**, Kaseno S, Takada N, Takemura M, Kisida S, Okuda E, Ueno M, Tanaka Y, Fukuhara K, Kinoshita H. [Clinical significance of ambulatory intraesophageal bilirubin monitoring in diagnosis of gastroesophageal reflux] *Nippon Rinsho* 2000; **58**: 1823-1826

10 **Tack J**, Koek G, Demedts I, Sifrim D, Janssens J. Gastroesophageal reflux disease poorly responsive to single-dose proton pump inhibitors in patients without Barrett's esophagus: acid reflux, bile reflux, or both? *Am J Gastroenterol* 2004; **99**: 981-988

11 **Lundell LR**, Dent J, Bennett JR, Blum AL, Armstrong D, Galmiche JP, Johnson F, Hongo M, Richter JE, Spechler SJ, Tytgat GN, Wallin L. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut* 1999; **45**: 172-180

12 **Lee YC**, Wang HP, Chiu HM, Liao SC, Huang SP, Lai YP, Wu MS, Chen MF, Lin JT. Comparative analysis between psychological and endoscopic profiles in patients with gastroesophageal reflux disease: a prospective study based on screening endoscopy. *J Gastroenterol Hepatol* 2006; **21**: 798-804

13 **Maekawa T**, Kinoshita Y, Okada A, Fukui H, Waki S, Hassan S, Matsushima Y, Kawanami C, Kishi K, Chiba T. Relationship between severity and symptoms of reflux oesophagitis in elderly patients in Japan. *J Gastroenterol Hepatol* 1998; **13**: 927-930

14 **Okamoto K**, Iwakiri R, Mori M, Hara M, Oda K, Danjo A, Ootani A, Sakata H, Fujimoto K. Clinical symptoms in endoscopic reflux esophagitis: evaluation in 8031 adult subjects. *Dig Dis Sci* 2003; **48**: 2237-2241

15 **Iwakiri K**, Hayashi Y, Kotoyori M, Sugiura T, Kawakami A, Sakamoto C. The minimum pressure of the lower esophageal sphincter, determined by the rapid pull-through method, is an index of severe reflux esophagitis. *J Gastroenterol* 2004; **39**: 616-620

16 **Somani SK**, Ghoshal UC, Saraswat VA, Aggarwal R, Misra A, Krishnani N, Naik SR. Correlation of esophageal pH and motor abnormalities with endoscopic severity of reflux esophagitis. *Dis Esophagus* 2004; **17**: 58-62

17 **Oh DS**, Hagen JA, Fein M, Bremner CG, Dunst CM, Demeester SR, Lipham J, Demeester TR. The impact of reflux composition on mucosal injury and esophageal function. *J Gastrointest Surg* 2006; **10**: 787-796; discussion 796-797

18 **Kivilaakso E**, Fromm D, Silen W. Effect of bile salts and related compounds on isolated esophageal mucosa. *Surgery* 1980; **87**: 280-285

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Does clamping during liver surgery predispose to thrombosis of the hepatic veins? Analysis of 210 cases

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Abstract

AIM: To test whether clamping during liver surgery predisposes to hepatic vein thrombosis.

METHODS: We performed a retrospective analysis of 210 patients who underwent liver resection with simultaneous inflow and outflow occlusion. Intraoperatively, flow in the hepatic veins was assessed by Doppler ultrasonography during the reperfusion phase. Postoperatively, patency of the hepatic veins was assessed by contrast-enhanced CT angiography, when necessary after 3-6 mo follow up.

RESULTS: Twelve patients (5.7%) developed intraoperative liver remnant swelling. However, intraoperative ultrasonography did not reveal evidence of hepatic vein thrombosis. In three of these patients a kinking of the common trunk of the middle and left hepatic veins hindering outflow was recognized and was managed successfully by suturing the liver remnant to the diaphragm. Twenty three patients (10.9%) who developed signs of mild outflow obstruction postoperatively, had no evidence of thrombi in the hepatic veins or flow disturbances on ultrasonography and contrast-enhanced CT angiography, while hospitalized. Long term assessment of the patency of the hepatic veins over a 3-6 mo follow-up period did not reveal thrombi formation or clinical manifestations of outflow obstruction.

CONCLUSION: Extrahepatic dissection and clamping of the hepatic veins does not predispose to clinically important thrombosis.

Key words: CT-angiography; Doppler ultrasound; Liver resection; Pringle maneuver; Radiofrequency; Selective hepatic vascular exclusion

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INTRODUCTION

Vascular control during liver resection typically involves hepatic inflow occlusion, either continuous or intermittent. However, more complex resections may require both inflow and outflow occlusion, the latter usually being achieved with extraparenchymal control of the major hepatic veins at the hepatocaval junction. This maneuver can significantly reduce backflow bleeding during parenchymal transection and facilitate resection

of tumors close to the roots of the major hepatic veins and even reconstruction of a hepatic vein in the liver remnant[1].

Three techniques for vascular control have been widely used: the Pringle maneuver (PM), the selective hepatic vascular exclusion (SHVE) and the total hepatic vascular exclusion (THVE). The PM[2] is performed by encircling and clamping the hepatoduodenal ligament[1]. Although this is well tolerated, it should always be kept in mind that backflow bleeding and air embolization might occur during parenchymal transection[3]. THVE includes clamping of the hepatoduodenal ligament and occlusion of the suprahepatic and infrahepatic inferior vena cava (IVC)[4]. This technique has the advantage of a bloodless surgical field, but the serious hemodynamic instability it may cause, makes it inappropriate for 20%-30% of patients. SHVE entails disconnection of the liver from the retrohepatic IVC and inflow occlusion combined with extrahepatic control of hepatic veins. This technique offers bloodless liver transection without the above-mentioned disadvantages of PM and THVE[5,6].

Dissection and clamping of the hepatic veins during the application of SHVE may predispose the major hepatic veins to thrombi formation through the induction of venous stasis and endothelial injury coupled with coagulation disturbances. The scarcity of studies addressing this issue prompted us to test our hypothesis that dissection and clamping of the hepatic veins is associated with an increased risk of thrombosis and liver outflow obstruction.

MATERIALS AND METHODS

Between 1997 and 2007, 210 consecutive patients underwent hepatectomy with SHVE[1]. Briefly, in all cases and irrespective of the type of planned hepatectomy, the abdomen was accessed *via* a bilateral subcostal incision and the liver was fully mobilized after transection of its ligaments. The liver was then disconnected from the retrohepatic IVC by dividing the short perforator hepatic veins. On the right side, dissection along the anterior surface of the IVC continued until the right hepatic vein was isolated, while on the left side, the venous trunks of the left and middle hepatic veins were also dissected free from the surrounding tissues. Control of liver inflow was attained by clamping the porta hepatis with a Satinsky clamp and by occluding any accessory hepatic artery with bulldog clamps. Liver outflow control was achieved by clamping the trunks of the right, middle and left hepatic veins separately (Figure 1). The transection plane was defined with an intraoperative ultrasonographer (Aloka SSD-1400, model IP-1235V, ALOKA CO., LTD., Japan), in order to secure tumor-free margins > 1 cm. Liver splitting was performed using either the clamp crushing technique or by sharp transection with a knife. Hemostasis was achieved by suturing all vascular orifices on the cut surface with 3-0 and 4-0 prolene. After completion of the liver resection, outflow was released first, followed by liver inflow[5,6]. Following reperfusion, hemostasis was completed using additional stitches. Flow in the hepatic artery, the portal vein, the hepatic veins and the IVC was assessed by intraoperative Doppler ultrasonography. The negative findings in the first 120 consecutive patients prompted us to restrict our Doppler study to the hepatic veins. Thorough imaging of all liver vasculature was then reserved for cases with liver remnant swelling or signs of hyporeperfusion. All operations were performed by the same surgical team, directed by the senior author V.S. All patients had normal imaging of the hepatic veins before liver resection, either on contrast-enhanced CT or MRI.

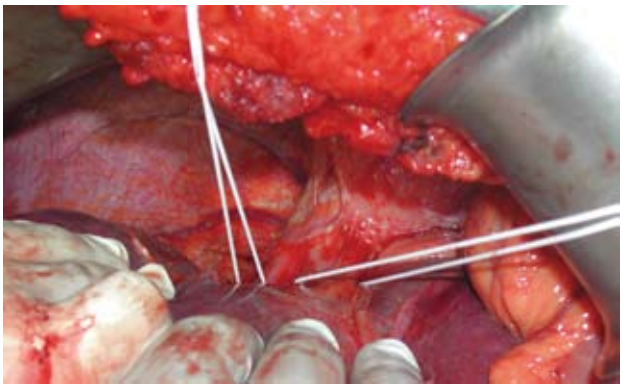

Figure 1 After careful dissection at the hepatocaval junction, the extrahepatic trunk of the right hepatic vein and the common trunk of the left and middle hepatic vein have been isolated and are ready to be clamped.

Postoperative Doppler ultrasonography of the portal vein, hepatic artery and the hepatic veins was performed at the bedside, if the patient exhibited at least one of the following findings: (a) clinically worsening ascites on any postoperative day; (b) persistent or worsening cholestasis (conjugated bilirubin > 3 mg/dL) after the 3rd postoperative day; (c) worsening elevation of transaminases after the 2nd postoperative day (AST and/or ALT > 1st postoperative day levels) or (d) persistent or worsening prolongation of INR > 2 after the 3rd postoperative day.

All patients admitted after 2002 (117 patients-56%) received perioperative thromboprophylaxis with low molecular weight heparin for a median duration of 10 d (range: 4-14 d), according to our new institutional protocol which was initiated at that time. Post-discharge, all patients were invited for a follow-up abdominal CT scan, 3-6 mo after surgery. Helical CT (Philips, The Netherlands) of the abdomen was performed before and after intravenous administration of iodinated contrast material. Contrast enhanced images were obtained in the arterial and portal venous phase and images were evaluated for the presence of hepatic venous thrombosis by two independent observers. Data collection was performed in a prospective manner.

RESULTS

Clinical, intraoperative and postoperative parameters of the patients are summarized in Table 1. Intraoperatively, 12 (5.7%) patients developed unexpected swelling of the liver remnant. Intraoperative Doppler ultrasonography

Table 1 Clinical, intraoperative and postoperative parameters of 210 patients undergoing liver resections under selective hepatic vascular exclusion (data are expressed as medians with range)

Clinical, intraoperative and postoperative parameters	
Age (yr)	63 (1-86)
Indication for hepatectomy	
Malignancy	173
Benign (hemangioma, hydatid cyst, *etc*)	37
Type of hepatectomy	
Right hepatectomy	92
Left hepatectomy	40
Minor hepatectomy (1-2 segments)	78
Intraoperative data	
Warm ischemic time (min)	39 (25-61)
Blood loss (mL)	420 (140-3100)
Transfusions of pRBCs (U)	0 (0-14)
Outcome	
Morbidity (pleural effusion, bile leak, chest infection, post-operative bleeding, wound infection, postoperative liver failure)	34%
Mortality	3 (1.5%)[1]
Hospital stay (d)	12 (4-37)

[1]One death due to postoperative liver failure, 1 due to cardiomyopathy and cardiac arrest and 1 due to postoperative sepsis.

did not reveal evidence of hepatic vein thrombosis in any of these cases. In three of these patients kinking of the common trunk of the middle and left hepatic veins was recognized and was managed by suturing the liver remnant to the diaphragm. In the remaining nine patients, extensive work-up did not show thrombi formation and the liver remnant swelling was attributed to the fact that the portal flow was disproportional to the small liver remnant.

Twenty three (10.9%) patients fulfilled the previously mentioned criteria for postoperative Doppler ultrasonography at the bedside. All these patients were also examined with contrast enhanced CT-angiography. The hepatic veins were visualized in all cases and no evidence of thrombosis was found.

Forty two (20%) patients underwent contrast-enhanced CT scans during their postoperative hospitalization for reasons unrelated to suspected hepatic vein thrombosis (diagnostic work-up for fever, bile collection and chest infection). In all cases, hepatic vein imaging did not reveal thrombi formation or recanalization processes.

Finally, 200 patients (95%) underwent a follow up contrast-enhanced CT scan of the liver at a median time of 150 d (range: 92-205 d) after surgery, without evidence of thrombotic processes or stricture of the hepatic veins.

DISCUSSION

Our study showed that dissection and clamping of the hepatic veins in hepatectomies under inflow and outflow vascular occlusion of the liver was not complicated with hepatic vein thrombosis either intraoperatively or postoperatively during a 6-mo follow-up period.

Postoperative hepatic venous outflow obstruction is extremely rare in large series of hepatectomies[7,8]. On the contrary, in liver transplantation the incidence is high, especially with the piggyback technique (0.5%-2.5%) which has a mortality rate of 24%[9-14]. The main causative factors are either technique-related or are associated with coagulation disturbances generated by the underlying disease and graft function.

The only study addressing the risk of hepatic vein thrombosis in liver resection is by Arita *et al*[15], who showed that 10 out of 821 liver resections performed using the intermittent Pringle maneuver developed hepatic vein thrombosis. It is worth noting that the authors had to resort to thrombectomy in the most severe cases. The exposure of major hepatic veins to a length of 3 cm or more and the use of an ultrasonic dissector were postulated to be predisposing factors for vein thrombosis.

Although our technique could be considered more thrombogenic, since it includes dissection and clamping of hepatic veins, the lack of thrombosis in our series is in surprising contrast to the findings of Arita *et al*, who performed only intermittent inflow occlusion. It is possible that the use of the ultrasonic dissection technique used by Arita *et al* could, as the authors themselves admitted, have contributed to the thrombogenic effect, which was further aggravated when the energy was delivered close to the major hepatic veins[15].

Our results are in agreement with the findings of most major clinical series of hepatectomies performed under vascular control, in which hepatic venous thrombosis is scarcely if ever mentioned[16,17]. Although SHVE could be considered more thrombogenic, since it involves injurious manipulation of hepatic veins, the lack of confirmed cases of vein thrombosis in our study can be attributed to short warm ischemic time and sharp transection of the liver surface with the scalpel, a technique that is less traumatic to venous epithelium compared to other ablative techniques. Avoidance of radiofrequency ablation in our series may also have contributed to our favorable results, since this technique has been recently associated with damage to the liver remnant[18] and hepatic vein thrombosis[19]. Venous endothelial trauma has been known to cause platelet aggregation and degranulation, vasoconstriction, thrombin activation and diminished fibrinolysis[20]. Therefore, we can not exclude the possibility that some of our patients may have developed small, undetected thrombi postoperatively. However, such thrombi remain clinically silent and resolve spontaneously without increasing morbidity or mortality. It is also possible that ischemia reperfusion of the liver mobilizes mechanisms that attenuate thrombi formation locally. Studies addressing the coagulation-fibrinolysis system during liver resection indicate that the balance leans towards fibrinolysis[21,22].

Regarding diagnosis of hepatic venous thrombosis, Doppler ultrasound is a readily available and inexpensive tool[23,24]. It is, however, operator-dependent and

its diagnostic accuracy may be compromised by the presence of bowel gas or ascites. On CT, vein thrombosis is seen as a lack of enhancement on post-contrast images, often associated with peripheral rim enhancement. In hepatic veno-occlusive disease, CT reveals patchy hepatic parenchymal enhancement with lack of normal visualization of the hepatic veins[25-27].

In conclusion, our analysis of a large cohort of patients confirms that extrahepatic dissection and clamping of the hepatic veins for up to one hour is a safe procedure that does not predispose to clinically important thrombosis. Although the technique of selective vascular exclusion used in our series is not advocated for routine use in liver surgery, we suggest that concerns about venous thrombosis are unjustified and should not be a limiting factor in the application of this useful technique, whenever necessary.

COMMENTS

Background

Dissection and clamping of the hepatic veins during liver resection may predispose the major hepatic veins to thrombi formation. In this study we test our hypothesis that dissection and clamping of the hepatic veins is not associated with an increased risk of thrombosis and liver outflow obstruction.

Research frontiers

Postoperative hepatic venous outflow obstruction is extremely rare in large series of hepatectomies. On the contrary, in liver transplantation the incidence is high, especially with the piggyback technique (0.5%-2.5%) which has a mortality rate of 24%. The main causative factors are either technique-related or are associated with coagulation disturbances generated by the underlying disease and graft function.

Innovations and breakthroughs

The only study addressing the risk of hepatic vein thrombosis in liver resection is by Arita *et al*, who showed that 10 out of 821 liver resections performed with the intermittent Pringle maneuver developed hepatic vein thrombosis. Our study showed that dissection and clamping of the hepatic veins in hepatectomies under inflow and outflow vascular occlusion of the liver was not complicated with hepatic vein thrombosis either intraoperatively or postoperatively during a six-month follow-up period.

Applications

Although the technique of selective vascular exclusion used in our series is not advocated for routine use in liver surgery, the authors suggest that concerns about venous thrombosis are unjustified and should not be a limiting factor in the application of this useful technique, whenever necessary.

Terminology

Pringle maneuver: performed by encircling and clamping the hepatoduodenal ligament; Selective hepatic vascular exclusion: inflow occlusion combined with extrahepatic control of hepatic veins; Total hepatic vascular exclusion: clamping of the hepatoduodenal ligament and occlusion of the suprahepatic and infrahepatic vena cava (IVC).

Peer review

This is an interesting paper about hepatic vein thrombosis.

REFERENCES

1 **Smyrniotis V**, Farantos C, Kostopanagiotou G, Arkadopoulos N. Vascular control during hepatectomy: review of methods and results. *World J Surg* 2005; **29**: 1384-1396

2 **Pringle JH**. Notes on the arrest of hepatic hemorrhage due to trauma. *Ann Surg* 1908; **48**: 541-549

3 **Hatano Y**, Murakawa M, Segawa H, Nishida Y, Mori K. Venous air embolism during hepatic resection. *Anesthesiology* 1990; **73**: 1282-1285

4 **Huguet C**, Addario-Chieco P, Gavelli A, Arrigo E, Harb J, Clement RR. Technique of hepatic vascular exclusion for extensive liver resection. *Am J Surg* 1992; **163**: 602-605

5 **Smyrniotis VE**, Kostopanagiotou GG, Contis JC, Farantos CI, Voros DC, Kannas DC, Koskinas JS. Selective hepatic vascular exclusion versus Pringle maneuver in major liver resections: prospective study. *World J Surg* 2003; **27**: 765-769

6 **Smyrniotis VE**, Kostopanagiotou GG, Gamaletsos EL, Vassiliou JG, Voros DC, Fotopoulos AC, Contis JC. Total versus selective hepatic vascular exclusion in major liver resections. *Am J Surg* 2002; **183**: 173-178

7 **Pan ZY**, Yang Y, Zhou WP, Li AJ, Fu SY, Wu MC. Clinical application of hepatic venous occlusion for hepatectomy. *Chin Med J* (Engl) 2008; **121**: 806-810

8 **Gurusamy KS**, Kumar Y, Sharma D, Davidson BR. Methods of vascular occlusion for elective liver resections. *Cochrane Database Syst Rev* 2007; CD006409

9 **Wang SL**, Sze DY, Busque S, Razavi MK, Kee ST, Frisoli JK, Dake MD. Treatment of hepatic venous outflow obstruction after piggyback liver transplantation. *Radiology* 2005; **236**: 352-359

10 **Ng SS**, Yu SC, Lee JF, Lai PB, Lau WY. Hepatic venous outflow obstruction after piggyback liver transplantation by an unusual mechanism: report of a case. *World J Gastroenterol* 2006; **12**: 5416-5418

11 **Perkins J**. Hepatic venous outflow obstruction after piggyback orthotopic liver transplantation. *Liver Transpl* 2006; **12**: 159-160

12 **Nishida S,** Nakamura N, Vaidya A, Levi DM, Kato T, Nery JR, Madariaga JR, Molina E, Ruiz P, Gyamfi A, Tzakis AG. Piggyback technique in adult orthotopic liver transplantation: an analysis of 1067 liver transplants at a single center. *HPB* (Oxford) 2006; **8**: 182-188

13 **Sze DY**, Semba CP, Razavi MK, Kee ST, Dake MD. Endovascular treatment of hepatic venous outflow obstruction after piggyback technique liver transplantation. *Transplantation* 1999; **68**: 446-449

14 **Pitre J**, Panis Y, Belghiti J. Left hepatic vein kinking after right hepatectomy: a rare cause of acute Budd-Chiari syndrome. *Br J Surg* 1992; **79**: 798-799

15 **Arita J**, Kokudo N, Hasegawa K, Sano K, Imamura H, Sugawara Y, Makuuchi M. Hepatic venous thrombus formation during liver transection exposing major hepatic vein. *Surgery* 2007; **141**: 283-284

16 **Kimura F**, Miyazaki M, Suwa T, Sugiura T, Shinoda T, Itoh H, Nakagawa K, Ambiru S, Shimizu H, Yoshitome H. Evaluation of total hepatic vascular exclusion and pringle maneuver in liver resection. *Hepatogastroenterology* 2002; **49**: 225-230

17 **Zhou W**, Li A, Pan Z, Fu S, Yang Y, Tang L, Hou Z, Wu M. Selective hepatic vascular exclusion and Pringle maneuver: a comparative study in liver resection. *Eur J Surg Oncol* 2008; **34**: 49-54

18 **Mitsuo M**, Takahiro T, Yasuko T, Masayasu A, Katsuya O, Nozomi S, Yoshihide O, Isamu K. Radiofrequency (RF)-assisted hepatectomy may induce severe postoperative liver damage. *World J Surg* 2007; **31**: 2208-2212; discussion 2213-2214

19 **Akahane M**, Koga H, Kato N, Yamada H, Uozumi K, Tateishi R, Teratani T, Shiina S, Ohtomo K. Complications of percutaneous radiofrequency ablation for hepatocellular carcinoma: imaging spectrum and management. *Radiographics* 2005; **25** Suppl 1: S57-S68

20 **Chung I**, Lip GY. Virchow's triad revisited: blood constituents. *Pathophysiol Haemost Thromb* 2003; **33**: 449-454

21 **Meijer C**, Wiezer MJ, Hack CE, Boelens PG, Wedel NI, Meijer S, Nijveldt RJ, Statius Muller MG, Wiggers T, Zoetmulder FA, Borel Rinkes IH, Cuesta MA, Gouma DJ, van de Velde CJ, Tilanus HW, Scotte M, Thijs LG, van Leeuwen PA. Coagulopathy following major liver resection: the effect of rBPI21 and the role of decreased synthesis of regulating proteins by the liver. *Shock* 2001; **15**: 261-271

22 **Tsuji K**, Eguchi Y, Kodama M. Postoperative

hypercoagulable state followed by hyperfibrinolysis related to wound healing after hepatic resection. *J Am Coll Surg* 1996; **183**: 230-238

23 **Boozari B**, Bahr MJ, Kubicka S, Klempnauer J, Manns MP, Gebel M. Ultrasonography in patients with Budd-Chiari syndrome: diagnostic signs and prognostic implications. *J Hepatol* 2008; **49**: 572-580

24 **Chong WK**, Beland JC, Weeks SM. Sonographic evaluation of venous obstruction in liver transplants. *AJR Am J Roentgenol* 2007; **188**: W515-W521

25 **Karaosmanoglu D**, Karcaaltincaba M, Akata D, Ozmen M, Akhan O. CT, MRI, and US findings of incidental segmental distal hepatic vein occlusion: a new form of Budd-Chiari syndrome? *J Comput Assist Tomogr* 2008; **32**: 518-522

26 **Lupescu IG**, Dobromir C, Popa GA, Gheorghe L, Georgescu SA. Spiral computed tomography and magnetic resonance angiography evaluation in Budd-Chiari syndrome. *J Gastrointestin Liver Dis* 2008; **17**: 223-226

27 **Meng XC**, Zhu KS, Qin J, Zhang JS, Wang XH, Zou Y, Zhang YQ, Shan H. Clinical significance of multislice spiral CT scans in hepatic veins occlusion in Budd-Chiari syndrome. *Chin Med J* (Engl) 2007; **120**: 100-105

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BRIEF ARTICLES

A study of pulmonary embolism after abdominal surgery in patients undergoing prophylaxis

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Abstract

AIM: To determine risk factors for pulmonary embolism and estimate effects and benefits of prophylaxis.

METHODS: We included 78 patients who died subsequently to a pulmonary embolism after major abdominal surgery from 1985 to 2003. A first, retrospective analysis involved 41 patients who underwent elective surgery between 1985 and 1990 without receiving any prophylaxis. In the prospectively evaluated subgroup, 37 patients undergoing major surgery between 1991 and 2003 were enrolled: all of them had received a prophylaxis consisting in low-molecular weight heparin, given subcutaneously at a dose of 2850 IU AXa/0.3 mL (body weight < 50 kg) or 5700 IU AXa/0.6 mL (body weight ⩾ 50 kg).

RESULTS: A higher incidence of thromboembolism (43.9% and 46.34% in the two groups, respectively) was found in older patients (> 60 years). The incidence of pulmonary embolism after major abdominal surgery in patients who had received the prophylaxis was significantly lower compared to the subjects with the same condition who had not received any prophylaxis ($P < 0.001$, OR = 2.825; 95% CI, 1.811-4.408). Furthermore, the incidence of pulmonary embolism after colorectal cancer surgery was significantly higher compared to incidence of pulmonary embolism after other abdominal surgical procedures. Finally, the incidence of pulmonary embolism after colorectal cancer surgery among the patients who had received the prophylaxis (11/4316, 0.26%) was significantly lower compared to subjects undergoing a surgical procedure for the same indication but without prophylaxis (10/1562, 0.64%) ($P < 0.05$, OR = 2.522; 95% CI, 1.069-5.949).

CONCLUSION: Prophylaxis with low molecular weight heparin is highly recommended during the preoperative period in patients with diagnosis of colorectal cancer due to high risk of pulmonary embolism after elective surgery.

Key words: Pulmonary embolism; Surgery; Colorectal cancer; Risk factor; Prevention

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INTRODUCTION

Pulmonary embolism (PE) is a life-threatening condition or complication and might be one of the worst nightmares for most surgeons. PE is a partial obstruction of the pulmonary arterial three. The embolus that causes the obstruction usually travels through the venous system from a distant site. PE causes symptoms such as dyspnea, chest pain or collapse. Moreover, the clinical severity of PE can vary, ranging from asymptomatic cases to sudden death. Despite advances in diagnosis and treatment, PE remains a significant cause of morbidity and mortality and is still one of the most common preventable causes of

death, which is easily overlooked[1,2].

Risk factors for deep vein thrombosis (DVT) and PE are prior medical history of DVT or PE, recent surgery, general anesthesia lasting longer than 30 min, pregnancy, prolonged immobilization, age > 40 years, obesity or underlying malignancy[3,4]. Moreover, gynecologic surgery, major trauma and indwelling venous catheters are risk factors for DVT at any location. Otherwise, venous thrombosis commonly involves lower limbs, affecting most frequently calf veins, which are involved in virtually 100% of symptomatic, spontaneous lower extremity DVT. Although DVT usually starts in calf veins, it is propagated above the knee in 87% of symptomatic patients before the diagnosis has been made. However, more than 35% of patients who die from PE may have isolated calf vein thrombosis[5].

MATERIALS AND METHODS

Subjects

We identified 54690 patients who had surgery between January, 1985 and December, 2003. The study included 39 (50%) females and 39 (50%) males who died subsequently to a PE after major abdominal surgery throughout the study period.

Retrospective analysis

The database of the Institute for Digestive Disease, Clinical Center of Serbia was reviewed to identify patients who had undergone surgery from January, 1985 to December, 1990. Age, final pathologic diagnosis at authopsy, surgical procedures and venous thromboembolism prophylaxis modalities were recorded.

In a first retrospective evaluation, out of the 15427 patients who had undergone surgery between 1985 and 1990, we included 41 cases (20 men, 21 women) of PE confirmed at autopsy. These patients had not received any prophylaxis prior to elective surgery, as prophylaxis was not performed on a regular basis during that period in our country. The PE patients had undergone the following surgical procedures: total oesophagogastrectomy, 1 case (2,4%); total gastrectomy, 3 (7.3%); Billroth I -type gastric resection, 2 (4.8%); Billroth II-type gastric resection, 4 (9.7%); gastrostomy with jejunostomy, 1 (2.4%); fundoplication, 1 (2.4%); cholecystectomy, 5 (12.1%); ileal resection, 1 (2.4%); nephrectomy, 1 (2.4%); appendectomy, 2 (4.8%); inguinal hernioplasty, 2 (4.8%); adhesiolysis, 1 (2.4%); laparotomic exploration with biopsy of the tumor, 5 (14.6%); right hemicolectomy, 1 (2.4%); left hemicolectomy, 4 (9.7%); coecostomy, 1 (2.4%); splenectomy with distal pancreatectomy, 1 (2.4%); and low anterior resection of the rectum, 3 (7.32%).

Prospective analysis

In the second part of our study, a total of 39263 patients were admitted to the Institute of Digestive Disease, Clinical Center of Serbia, between January, 1991 and July, 2003, to undergo abdominal elective surgery. All patients who underwent major surgery received low-molecular weight heparin (LMWH) prophylaxis subcutaneously at a dose of 2850 IU AXa/0.3 mL (body weight < 50 kg) or 5700 IU AXa/0.6 mL (body weight ⩾ 50 kg), one hour before the surgery, 12 and 24 h after the main surgery, and once daily each hospital day after main surgery. Prophylaxis did not cause any side effects (e.g. bleeding). Out of this group, a total of 37 patients (19 men, 18 women) died after major surgery due to PE. Diagnosis was confirmed at autopsy. These patients had undergone the following major surgery procedures: total gastrectomy, 5 (13.5%); ulcer suture, 2 (5.4%); gastrostomy, 2 (5.4%); cholecystectomy, 4 (10.8%); hepatico-jejuno anastomosis according to Roux, 2 (5.4%); partial pericystectomy with omentoplasty, 2 (5.4%); ileal resection, 1 (2.7%); appendectomy, 1 (2.7%); hernioplasty, 1 (2.7%); abscess drainage, 1 (2.7%); laparotomic exploration with biopsy of the tumor, 2 (5.4%); total colectomy, 2 (5.4%); right hemicolectomy, 3 (8.1%); left hemicolectomy, 3 (8.1%), Dixon-type resection, 2 (5.4%); Hartman-type resection, 2 (5.4%); Belsey-type resection, 1 (2.7%) and double colostomy, 1 (2.7%) (Table 1).

Statistical analysis

Results were presented as mean ± SD or as stated. Distribution data were compared by χ^2 analysis or Kruskal-Wallis test, if data were not normally distributed. In addition, logistic regression tests were conducted. All statistical analyses were performed with the SPSS 10.0 for Windows package (SPSS Inc., Chicago, IL). Values at the $P = 0.05$ level were considered statistically significant.

RESULTS

No significant difference as to the gender distribution existed between the two groups (χ^2 test, $P > 0.05$). No significant difference in the mean age was found between patient groups (Kruskal-Wallis test, $P > 0.05$). However, a higher incidence of thromboembolism (43.9% and 46.34%) was found in older patients (60-69 year range) in both groups of patients.

Forty-one patients out of 15427 (0.27%) who did not receive prophylaxis developed a PE. Among the 39263 patients who received prophylaxis, 37 (0.09%) developed a PE in the postoperative period.

Among the 15427 cases evaluated retrospectively, we identified 4304 patients who underwent colorectal abdominal surgery. Of them, 1562 were cancer cases. Among colorectal cancer patients who underwent major abdominal surgery, 0.64% (10/1562) developed PE postoperatively, while the incidence of PE in all remaining patients was 0.11% (16/13865) ($P < 0.05$, OR = 5.577, 95% CI, 2.526-12.311).

Among the 39263 patients who had received prophylaxis before major surgery, 37 (0.09%) were diagnosed as having postoperative PE. Of the 39263 major cases evaluated prospectively, we identified 11735 patients who underwent colorectal abdominal surgery.

Table 1 Characteristics of patients with pulmonary embolism

Clinical data	PE without prophylaxis (n = 41)	PE with prophylaxis (n = 37)
Mean age, yr (range)	64 (26-79)	67 (45-79)
M/F	20/21	19/18
Cause of death (primary)		
Obstruction of right pulmonary artery	10	5
Obstruction of left pulmonary artery	3	5
Obstruction of pulmonary trunk	10	1
Obstruction of both right and left pulmonary arteries	11	20
Obstruction of pulmonary trunk plus both right and left pulmonary arteries	7	6
Cause of death (secondary)		
Colorectal malignancy	11	10
Other malignancy	14	12
Other diagnosis	16	15
Prophylaxis	None	LMWH *s.c*: 0.3 mL (BW ≤ 50 kg) or 0.6 mL (BW > 50 kg), 1 h before and 12 h after surgery
Time from surgery to death		
0-5 d	12	22
6-10 d	19	8
11-15 d	6	2
16-30 d	4	3
> 30 d	0	2

PE: Pulmonary embolism; LMWH: Low-molecular weight heparin.

Of them, 4316 were cancer cases. Among colorectal cancer patients who underwent major abdominal surgery, 0.25% (11/4316) developed PE postoperatively, while the incidence of PE in all remaining patients was 0.05% (17/34 947) ($P < 0.05$, OR = 5.250, 95% CI, 2.457-11.216).

The incidence of PE after colorectal cancer surgery among patients who had received prophylaxis was significantly lower compared to that observed in subjects with colorectal surgery due to carcinoma who had not received any prophylaxis, i.e. 0.26% (11/4316) *vs* 0.64% (10/1562) ($P < 0.05$, OR = 2.522; 95% CI, 1.069-5.949). Moreover, incidence of PE after major abdominal surgery of patients who had received prophylaxis was significantly lower compared to that seen in subjects with the same diagnoses who had not received any prophylaxis ($P < 0.001$, OR = 2.825; 95% CI, 1.811-4.408).

DISCUSSION

PE is third most common cause of death in the US, with at least 650 000 cases occurring annually. Furthermore, PE represents the first or second most common cause of unexpected death in most age groups. The highest incidence of recognized PE occurs in hospitalized patients. Autopsy results are showing that up to 60% of patients who die at a hospital have PE, and that diagnosis is missed in about 70% of cases[6]. The annual incidence of known DVT and PE in the Western world is 1.0 and 0.5 per 1000, respectively. There are 65 000 cases each year among hospital patients in England and Wales. The prevalence of unsuspected PE diagnosed at autopsy is 3%-8%, and has been unchanged for 3 decades.

PE is common during all trimesters of pregnancy and puerperium, and incidence of PE is increasing with oral contraceptive or hormone replacement therapy. However, sex alone is not an independent risk factor[7].

Although the frequency of PE increases with age, this is not independent risk factor. Nevertheless, the accumulation of different risk factors, such as underlying illnesses and decreased mobility, increases the frequency of PE in older patients. Unfortunately, diagnosis of PE is often missed, especially in older patients. PE is diagnosed in 30% of all patients who die with massive PE, but only in 10% of those who are 70 years of age or older. Thus, PE still remains the most commonly missed diagnosis in the elderly institutionalized patients[7].

In our study, we found a higher incidence of PE in older patients (> 60 years of age) in both groups (43.9% and 46.34%).

Surgical patients have long been recognized to be at special risk for DVT and PE, but these problems are not confined to surgical patients. Surgeons should always suspect PE in case of a sudden circulatory collapse occurring within one to two weeks after surgery.

The risk of postoperative venous thromboembolism is reported to be twice as high in patients with cancer compared of those without cancer undergoing comparable surgery[8]. This risk is also higher in patients undergoing surgery for colorectal cancer as compared to those having abdominal surgery without malignancy. Thromboembolic complications are responsible for about half of deaths following elective colorectal surgery[9]. The highest rate (1.8%) of fatal PE was reported in patients following colorectal surgery, with a 3.3-fold increase compared to the overall rate observed among surgical patients, according to a retrospective 10-year review from Switzerland[10]. In this study, the increased risk of PE can be explained by a number of factors, such as malignancy-related hypercoagulable

state, postoperative infectious complications, prolonged surgery, pelvic dissection *etc*[11].

Overall, the incidence of PE after general surgery observed in Japan was 0.33%. Fatal PE was reported in 0.08% of the surgical population and the mortality rate of patients with PE was 31%. In addition, the incidence of PE after cancer surgery ranged from 0.57% after colon malignancy to 3.85% after pancreatic cancer surgery, and was significantly higher than in non-cancerous conditions (0.20%)[12].

An increased risk of PE after colorectal surgery has also been showed by Lee *et al* in a study on Chinese patients who underwent colorectal surgery without DVT prophylaxis. The authors demonstrated the occurrence of asymptomatic calf vein thrombosis in 41.7% of patients using serial Duplex ultrasound studies[13].

In our experience, the incidence of PE after colorectal cancer surgery was significantly higher compared with other surgical procedures. However, the incidence of PE after colorectal cancer surgery of patients who received prophylaxis was significantly lower compared to that seen among subjects with colorectal surgery due to carcinoma without prophylaxis.

In the study by Shukla *et al*[11], 99 patients with colorectal cancer selected for surgery were included. Fifty-one patients were randomized to receive LMWH while 48 patients did not receive any prophylaxis. At the end of the study, neither DVT nor PE cases were observed[12].

Anticoagulant prophylaxis is effective in preventing PE in hospitalized patients, since it reduces mortality after surgery. Prophylaxis with LMWH leads to effective reductions in the incidence of DVT after abdominal surgery in patients at risk for thromboembolic complications.

Initial treatment with LMWH following oral anticoagulant therapy with INR ranging from 2 to 3 was associated with an incidence of major bleeding of 3% at 3 mo while the mortality rate was 0.3%[14].

However, Diener *et al*, showed that there may be a dose-dependent risk of bleeding with LMWH therapy[15]. Low dose of LMWH was arbitrarily defined as a fixed dose of less than 6000 IU daily, whereas any higher dose of LMWH was considered as LMWH high dose. Concerning weight-adjusted doses of LMWH, 86 IU/kg per day was considered as LMWH low dose, while 86 IU/kg twice a day was considered as LMWH high dose.

In our study, patients who received prophylaxis with low dose LMWH after major abdominal surgery did not have any side effects (such as bleeding). Moreover, incidence of PE was significantly lower compared to subjects with the same conditions who did not receive prophylaxis.

The incidence of PE was four to six times lower in patients who had mechanical prophylaxis, although the difference was not significant. Preoperative prophylaxis for DVT is important, but further research is needed to estimate its effects and benefits[12].

In our study, older age (> 60 years) was identified to be a risk factor for PE. Prophylaxis with LMWH is highly recommended for patients with colorectal cancer before major surgery. As the mortality from PE depends on correct and timely diagnosis, it is of the utmost importance for clinicians to consider this possibility and perform proper diagnostic tests, especially in patients with colorectal cancer.

COMMENTS

Background

Pulmonary embolism (PE) is a life-threatening condition or complication and might be also one of the worst nightmares for most surgeons. Despite advances in diagnosis and treatment, PE remains a significant cause of morbidity and mortality and is still one of the most common preventable causes of death, which is easily overlooked. Risk factors for deep vein thrombosis (DVT) and PE are prior medical history of DVT or PE, recent surgery, general anesthesia lasting longer than 30 min, pregnancy, prolonged immobilization, age > 40 years, obesity or underlying malignancy.

Research frontiers

PE is the third most common cause of death in the US, with at least 650 000 cases occurring annually. Furthermore, PE represents the first or second most common cause of unexpected death in most age groups. The highest incidence of recognized PE occurs in hospitalized patients. The highlight of this article was to characterize relationship between PE and prophylaxis with a low dose of low-molecular weight heparin.

Innovations and breakthroughs

The highest rate of fatal PE in previous studies was reported in patients following colorectal surgery. Shukla *et al* described that increased risk of PE has been attributed to a number of factors such as malignancy-related hypercoagulable state of cancer patients, postoperative complications due to infections, prolonged surgery and pelvic dissection. In our experience, incidence of PE after colorectal cancer surgery was also significantly higher compared with other surgical procedures. However, in our study, patients who received prophylaxis with low dose low-molecular weight heparin (LMWH) after major abdominal surgery did not have any side effects (such as bleeding). Moreover, the incidence of PE was significantly lower compared to subjects who did not receive the prophylaxis.

Applications

The results of this study suggest that prophylaxis with LMWH is highly recommended for older patients (> 60 years) and patients with colorectal cancer before major surgery. As the mortality from PE depends on a correct and timely diagnosis, it is of the utmost importance for clinicians to consider this possibility and perform proper diagnostic tests, especially in patients with colorectal cancer.

Terminology

Prophylaxis: A low dose of LMWH was arbitrarily defined as a fixed dose of less than 6000 IU daily. A dose of LMWH above 6000 IU was considered high dose LMWH. Concerning weight-adjusted doses LMWH, 86 IU/kg per day was considered as LMWH low dose while 86 IU/kg twice a day was considered LMWH high dose.

Peer review

This controlled study shows that prophylaxis with low dose of LMWH significantly decreases the incidence of PE after surgery. In addition, our research may foster new therapeutic developments in the treatment of PE.

REFERENCES

1 **Idiz M**, Konuralp C, Ates M. Under diagnosis of pulmonary embolism: a recurrent nightmare for surgeons. *Eastern J Med* 2003; **8**: 1-6
2 **Wolfe TR**, Hartsell SC. Pulmonary embolism: making sense of the diagnostic evaluation. *Ann Emerg Med* 2001; **37**: 504-514
3 **Robinson GV**. Pulmonary embolism in hospital practice. *BMJ* 2006; **332**: 156-160
4 **McAlister FA**, Bertsch K, Man J, Bradley J, Jacka M. Incidence of and risk factors for pulmonary complications after nonthoracic surgery. *Am J Respir Crit Care Med* 2005; **171**: 514-517
5 **Heit JA**. The epidemiology of venous thromboembolism in the community: implications for prevention and management. *J Thromb Thrombolysis* 2006; **21**: 23-29

6 **Klok FA**, Mos IC, Huisman MV. Brain-type natriuretic peptide levels in the prediction of adverse outcome in patients with pulmonary embolism: a systematic review and meta-analysis. *Am J Respir Crit Care Med* 2008; **178**: 425-430
7 **Konstantinides SV**. Massive pulmonary embolism: what level of aggression? *Semin Respir Crit Care Med* 2008; **29**: 47-55
8 **Prandoni P**. Antithrombotic strategies in patients with cancer. *Thromb Haemost* 1997; **78**: 141-144
9 **Huber O**, Bounameaux H, Borst F, Rohner A. Postoperative pulmonary embolism after hospital discharge. An underestimated risk. *Arch Surg* 1992; **127**: 310-313
10 **Wille-Jorgensen P**, Kjaergaard J, Jorgensen T, Korsgaard Larsen T. Failure in prophylactic management of thromboembolic disease in colorectal surgery. *Dis Colon Rectum* 1988; **31**: 384-386
11 **Shukla PJ**, Siddachari R, Ahire S, Arya S, Ramani S, Barreto SG, Gupta S, Shrikhande SV, Jagannath P, Desouza LJ. Postoperative deep vein thrombosis in patients with colorectal cancer. *Indian J Gastroenterol* 2008; **27**: 71-73
12 **Sakon M**, Kakkar AK, Ikeda M, Sekimoto M, Nakamori S, Yano M, Monden M. Current status of pulmonary embolism in general surgery in Japan. *Surg Today* 2004; **34**: 805-810
13 **Lee FY**, Chu W, Chan R, Leung YF, Liu KH, Ng SM, Lai PB, Metreweli C, Lau WY. Incidence of deep vein thrombosis after colorectal surgery in a Chinese population. *ANZ J Surg* 2001; **71**: 637-640
14 **Nijkeuter M**, Sohne M, Tick LW, Kamphuisen PW, Kramer MH, Laterveer L, van Houten AA, Kruip MJ, Leebeek FW, Buller HR, Huisman MV. The natural course of hemodynamically stable pulmonary embolism: Clinical outcome and risk factors in a large prospective cohort study. *Chest* 2007; **131**: 517-523
15 **Diener HC**, Ringelstein EB, von Kummer R, Langohr HD, Bewermeyer H, Landgraf H, Hennerici M, Welzel D, Grve M, Brom J, Weidinger G. Treatment of acute ischemic stroke with the low-molecular-weight heparin certoparin: results of the TOPAS trial. Therapy of Patients With Acute Stroke (TOPAS) Investigators. *Stroke* 2001; **32**: 22-29

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Usefulness of magnifying endoscopy in post-endoscopic resection scar for early gastric neoplasm: A prospective short-term follow-up endoscopy study

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Abstract

AIM: To investigate the relationship between post-endoscopic resection (ER) scars on magnifying endoscopy (ME) and the pathological diagnosis in order to validate the clinical significance of ME.

METHODS: From January, 2007 to June, 2008, 124 patients with 129 post-ER scar lesions were enrolled. Mucosal pit patterns on ME were compared with conventional endoscopy (CE) findings and histological results obtained from targeted biopsies.

RESULTS: CE findings showed nodular scars (53/129), erythematous scars (85/129), and ulcerative scars (4/129). The post-ER scars were classified into four pit patterns of sulci and ridges on ME: (Ⅰ) 47 round; (Ⅱ) 54 short rod or tubular; (Ⅲ) 19 branched or gyrus-like; and (Ⅳ) 9 destroyed pits. Sensitivity and specificity were 88.9% and 62.5%, respectively, by the presence of nodularity on CE. Erythematous lesions were high sensitivity (100%), but specificity was as low as 36.7%. The range of the positive predictive value (PPV) on CE was as low as 10.6%-25%. Nine type Ⅳ pit patterns were diagnosed as tumor lesions, and 120 cases of type Ⅰ-Ⅲ pit patterns revealed non-neoplastic lesions. Thus, the sensitivity, specificity, and the PPV of ME were 100%.

CONCLUSION: ME findings can detect the presence of tumor in post-ER scar lesions, and make evident the biopsy target site in short-term follow-up. Further large-scale and long-term studies are needed to determine whether ME can replace endoscopic biopsy.

Key words: Endoscopic mucosal resection; Endoscopic submucosal dissection; Magnifying endoscopy; Pit pattern; Scar

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INTRODUCTION

The use of magnifying endoscopy (ME) is now being reassessed since the successful study led by Professor Kudo regarding the utilization of magnifying colonoscopy[1]. Indeed, ME procedures for the upper gastrointestinal tract have been developed that make it possible to perform a variety of assessments, from routine

observation to a detailed examination of squamous dysplasia, squamous-cell carcinoma, Barrett's esophagus and associated dysplasia/early cancer, gastric cancer, and *Helicobacter pylori* infection[2-4]. ME with a narrow band image can aid in deciding the target of endoscopic biopsy for surveillance in Barrett's esophagus[5-8]. The relationships between ME findings and gastric neoplastic histology, including the types of cancer detected, are now being investigated, and the usefulness of ME for diagnosing early gastric cancer has been reported[9-15].

Little data, however, are currently available regarding the correlation between the findings of ME and pathological findings in post-endoscopic resection (ER) scars. There has been no definitive endoscopic description of which endoscopic findings need endoscopic biopsy or where the endoscopist has to target the biopsy in altered large scar lesions. In addition, it remains controversial as to whether a biopsy should be performed for each endoscopy in patients who have already undergone complete ER. Thus, we have evaluated the relationship between the real-time diagnosis of post-ER scars observed by ME and the pathological diagnosis, thereby validating the clinical usefulness of ME as a follow-up method for post-ER scars in early gastric neoplasm.

MATERIALS AND METHODS

Patients and definition

From January, 2007 to June, 2008, a total of 143 lesions (138 patients) underwent endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) in our hospital (Cheonan Hospital, Soonchunhyang University). "*En bloc* resection" is defined as the resection of a single piece as opposed to piecemeal resection in multiple pieces. "Complete *en bloc* resection" is defined as a lesion being contained within the mucosal layer, with no lympho-vascular invasion, and all margins (deep and lateral) histologically demonstrated to be tumor-free.

Among 138 patients, 8 patients who had been revealed as incomplete ER were excluded because they received a subsequent operation. Other exclusion criteria were as follows: (1) refusal to participate in the study (3 cases); (2) recurrent tumorous lesions (1 case); (3) NSAIDs or anticoagulant drug users (2 cases). A total of 129 lesions (124 patients) were finally enrolled in this study. All patients provided written informed consent, and the clinical study was performed according to guidelines approved by the ethics committee of Soonchunhyang Cheonan Hospital. No patients were lost during follow-up.

Methods

For the endoscopic examination, we used a GIF-Q240Z video endoscope (Olympus Optical Co., Ltd, Tokyo, Japan) fitted with an optic-type zoom lens that provided up to 80 times magnification and a high-resolution color charge-coupled device (CCD) connected to a 14-inch monitor. A transparent tip attachment (D-201-11802; Olympus) projecting 2 mm from the endoscopic tip was pressed against the mucosa in order to maintain good focus. To decrease the influence of mucus in the stomach under magnified observation, all patients ingested simethicone (20-30 mL), and a mucolytic agent (10% N-acetylcysteine 20 to 30 mL) was sprayed on the mucosal surface[4]. Evaluation of the entire stomach was initially performed with conventional endoscopy (CE) to exclude obvious lesions and to define scar lesions. Next, with the endoscope positioned on the scar lesions, complete magnification was obtained, with particular attention being paid to minute surface architecture and arrangements. Following complete identification, targeted biopsy specimens of the scar lesions were obtained. Conventional and magnifying endoscopic procedures were performed by an endoscopist with 10 years endoscopic experience. All examinations and images were digitally stored and documented on commercially available videotapes. Classification and analysis of the magnified view were carried out using the photographs and recorded videos by another endoscopist who was blinded to the examinations and histopathologic results. When pit patterns were mixed, classification was based on the most prominent pattern. We performed an endoscopic biopsy on sites with prominent or higher grade pit patterns. Following ER, all patients were given a PPI (omeprazole 40 mg) for eight weeks. Conventional and magnifying endoscopies were performed with the targeted biopsy of all scar lesions two months after the ER.

Classification by conventional and magnifying endoscopy

We classified CE characteristics of scar lesions according to the following attributes: height (elevated, flat, or depressed); nodularity (non-nodular or nodular); color (erythematous, pale, or iso-color with the surrounding mucosa); and ulceration (present or absent) (Figure 1). Next, the mucosal pit patterns in the post-ER scars were observed closely using ME. The mucosal pits were classified into four patterns of sulci and ridges: (Ⅰ) round pit patterns; (Ⅱ) short rod or tubular pit patterns; (Ⅲ) branched or gyrus-like pit patterns; and (Ⅳ) destroyed pit patterns (Figure 2). The criteria for suspecting a tumorous lesion included the observation of a fundamentally destroyed pit pattern (Type Ⅳ).

Histological assessment

The curative potential of *en bloc* resection was carefully evaluated histopathologically; slices were made at 2 mm intervals according to the Japanese Classification of Gastric Carcinoma[16]. Following magnifying observation, standard histological assessment was performed with H&E staining. Lesions were classified into four groups for diagnostic purposes: non-neoplastic lesions, low-grade adenomas, high-grade adenomas, and carcinomas. These diagnostic criteria were based on the Vienna classification of gastrointestinal epithelial neoplasia[17]. The histological type and the degree of various pathologic findings were evaluated to determine the relationship between endoscopic findings such as foveolar hyperplasia,

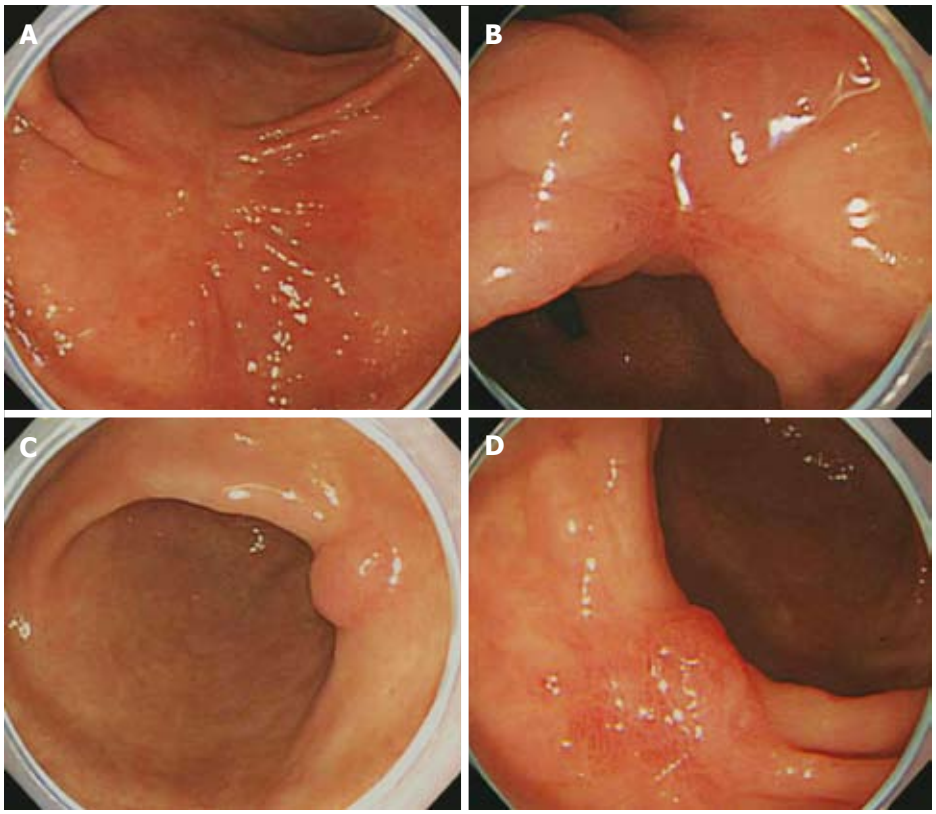

Figure 1 Conventional endoscopic characteristics of scar lesions. A: Height; flat, non-nodular and iso-color; B: Height; depressed, non-nodular and erythematous color; C: Height; elevated, non-nodular, and iso-color; D: Height; elevated, distorted nodular, and erythematous color.

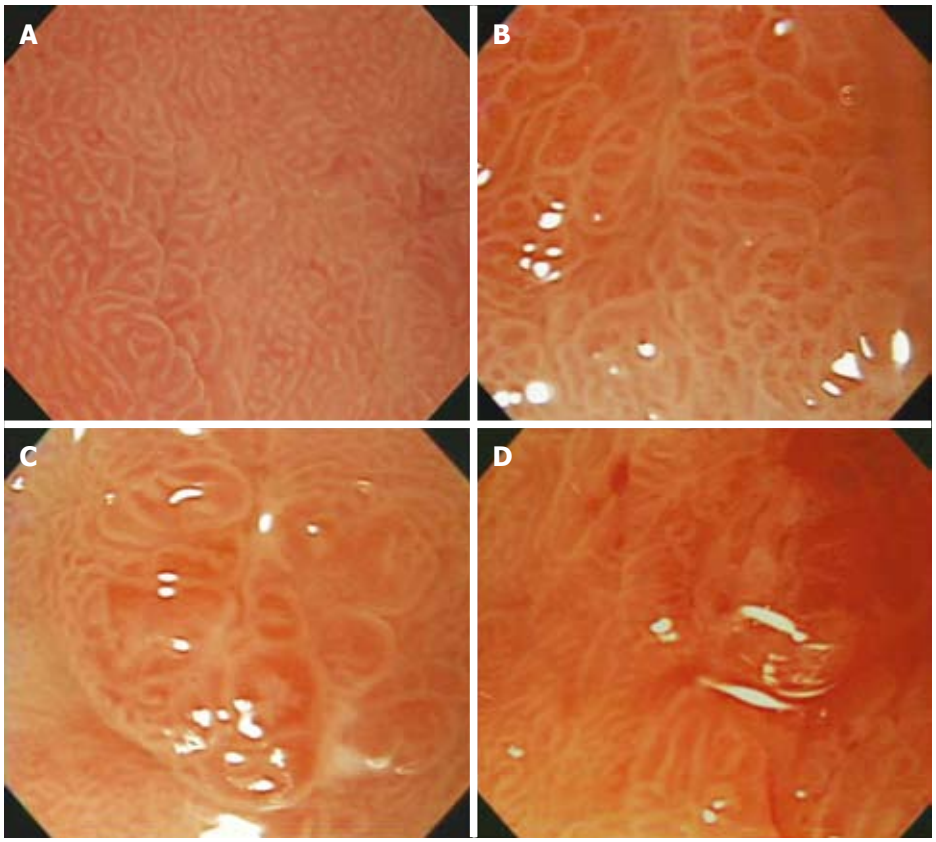

Figure 2 Magnifying images of scar lesion showing fine mucosal pit patterns. The four pit patterns of sulci and ridges identified are as follows: A: Type Ⅰ pit; small round, normal-like pit pattern; B: Type Ⅱ pit; short rod or tubular pit pattern; C: Type Ⅲ pit; branched or gyrus-like pit pattern; D: Type Ⅳ pit; destroyed pit pattern.

congestion of glands, atrophy, intestinal metaplasia, and fibrosis. These findings were then classified and scored from 0 to 3, respectively (0 = normal, 1 = mild, 2 = moderate, 3 = severe). A single pathologist who was blinded to the endoscopic findings reviewed and scored all the biopsy specimens. All pathologic findings were then compared in terms of both CE and ME findings.

Statistical analysis

Statistical evaluations were performed using SPSS

Table 1 Patient characteristics

Parameter	
Case (Patient)	129 (124)
Male	79
Female	45
Age, yr (SD)	58.51 (11.27)
Male	59.48 (11.79)
Female	56.71 (10.13)
ER outcome	
ESD/EMR	104/25
Complete resection	123
En bloc	117
Piecemeal	6
Incomplete resection	6
Post-ER diagnosis	
Adenoma	43 lesions
Adenoma with HGD	48 lesions
Adenocarcinoma	38 lesions
Follow up mo (SD)	2.27 (0.46)

SD: Standard deviation; ER: Endoscopic resection; ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; HGD: High grade dysplasia.

statistical software, version 12.0 (SPSS Inc., Chicago, IL, USA). The Chi-square test was used to compare categorical variables and the ANOVA test was used to compare continuous variables. A P value of less than 0.05 was considered statistically significant.

RESULTS

Outcomes and histological diagnosis of the ER

Among 129 lesions, the method of ER was 104 ESD and 25 EMR. Complete resection was performed in 123 cases [117 *en bloc* resections (93 ESD and 24 EMR) and 6 piecemeal resections (2 ESD and 4 EMR)]. Six patients with incomplete resection who declined surgery were included in a follow-up endoscopic study. A set of 129 lesions from 124 patients were confirmed histologically by ER as consisting of 38 adenocarcinomas, 48 high-grade adenomas, and 43 low-grade adenomas. Following ER, the mean follow-up time was 2.27 ± 0.46 mo (mean ± SD) (Table 1).

Conventional and magnifying findings in post-ER scars

The CE findings revealed the following results: 38 elevated, 79 flat, and 12 depressive-type scars; 76 non-nodular and 53 nodular scars; 85 erythematous and 44 pale or iso-colored scars; and 4 ulcerative scars. The minute surface structure of post-ER scars, as shown by ME, demonstrated four pit patterns of sulci and ridges. These pit patterns were classified according to the main pit pattern as follows: (Ⅰ) 47 round pit patterns; (Ⅱ) 54 short rod or tubular pit patterns; (Ⅲ) 19 branched or gyrus-like pit patterns; and (Ⅳ) 9 destroyed pit patterns. There was no statistical significance between conventional endoscopic and ME findings ($P > 0.05$), although the presence of nodularity and erythematous lesions was high in type Ⅲ or IV pit patterns on ME ($P = 0.091$, $P = 0.079$, respectively, Table 2).

Table 2 Endoscopic findings and pit pattern in post-ER scar

CE findings	Pit type (No.)				Total (129)	*P* value
	Ⅰ (47)	Ⅱ (54)	Ⅲ (19)	Ⅳ (9)		
Height						0.205
Elevated	14	20	3	6	38	
Flat	29	29	13	3	79	
Depressed	4	5	3	0	12	
Nodularity						0.091
Present	17	18	11	6	53	
Absent	30	36	8	3	76	
Color						0.079
Erythematous	30	31	16	8	85	
Iso or pale	17	23	3	1	44	
Ulceration						0.285
Present	0	2	1	1	4	
Absent	47	52	18	8	125	

CE: Conventional endoscopy; Ⅰ: Round pit; Ⅱ: Short rod or tubular pit; Ⅲ: Branched or gyrus-like pit; Ⅳ: Destroyed pit pattern.

Endoscopic findings and pathologic features

Eight lesions revealed the presence of tumors in 53 cases with nodularity, while one lesion had no nodularity in the post-ER scar. Nine lesions revealed the presence of tumors in 85 cases with erythematous lesions. One lesion revealed the presence of tumors in 4 cases with non-healed ulcer lesions. Sensitivity and specificity were 88.9% and 62.5%, respectively, when the presence of nodularity aided in the detection of a neoplastic lesion on CE. Erythematous lesions had a high sensitivity (100%), but specificity was as low as 36.7%. The presence of an ulcer had low sensitivity (11.1%) and high specificity (97.5%). The range of the positive predictive value was as low as 10.6%-25% (Table 3). As assessed by CE, none of the mucosal height terms, color, nodularity, or ulceration showed statistical significances between various non-neoplastic pathologic features in post-ER scars.

Although there was no statistical significance in the relationship between endoscopic findings and other non-neoplastic pathologic findings, type Ⅲ or Ⅳ pit patterns exhibited slightly higher histological scores in terms of gland congestion, intestinal metaplasia, and atrophy ($P > 0.05$, Table 4). Nine type Ⅳ pit patterns on ME were diagnosed as tumor lesions, pathologically, and 120 cases of type Ⅰ-Ⅲ pit patterns revealed non-neoplastic lesions without tumor lesions. Thus, the sensitivity, specificity, and the positive predictive value were 100%, 100% and 100%, respectively (Table 5). Six cases were noted in patients who had received incomplete resection. Three cases of piecemeal resection for early gastric cancer were diagnosed as tumor lesions in spite of histologically complete resection.

DISCUSSION

Magnifying colonoscopy has already been reported as a clinically useful tool for diagnosing colorectal tumors[1,18]. Furthermore, ME has been confirmed as being superior to conventional colonoscopy with respect to its predictive

Table 3 Endoscopic findings and pathologic result in post-ER scar

CE findings (No.)	Pathologic results					
	Non-neoplastic	Neoplastic	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
Presence of nodularity (53)	45	8	88.9 (68.4-100)	62.5 (53.8-71.2)	15.1 (5.5-24.7)	98.7 (96.1-100)
Erythematous lesion (85)	76	9	100.0	36.7 (28.0-45.3)	10.6 (4.0-17.1)	100.0
Presence of ulcer (4)	3	1	11.1 (0.0-31.6)	97.5 (94.7-100)	25.0 (0.0-67.4)	93.6 (89.3-97.9)
Total No.	120	9				

PPV: Positive predictive value; NPV: Negative predictive value; CE: Conventional endoscopy.

Table 4 Pit pattern and non-neoplastic pathologic findings (mean score) in post-ER scar

Pit type	Foveolar hyperplasia	Gland congestion	Intestinal metaplasia	Atrophy	Fibrosis
Ⅰ	0.96	0.64	1.09	1.11	0.21
Ⅱ	0.96	0.48	1.11	1.11	0.19
Ⅲ	0.84	0.79	1.05	1.26	0.11
Ⅳ (coexisting findings with tumor)	0.78	0.89	1.33	1.56	0.33
P value[1]	0.837	0.244	0.828	0.344	0.644

Scored from 0 to 3, respectively (0 = normal, 1 = mild, 2 = moderate, 3 = severe). [1]Statistical significances were tested by one-way analysis of variance.

Table 5 Pit pattern and pathologic result in post-ER scar

Pit type	Pathologic results	
	Non-neoplastic	Neoplastic
Ⅰ: Round	47	0
Ⅱ: Short rod or tubular	54	0
Ⅲ: Branched or gyrus-like	19	0
Ⅳ: Destroyed[1]	0	9
Total No.	120	9

[1]Sensitivity: 100%; Specificity: 100%; Positive predictive value: 100%.

power for diagnosing pathological neoplasms detected during endoscopy. Technological improvements in recent years have demonstrated that ME can identify the fine mucosal patterns of the gastrointestinal tract, and it is now evident that the findings obtained from this new procedure correlate positively with histological findings[19,20], and that ME can help determine the target biopsy site during surveillance in Barrett's esophagus[5-8]. Despite these advances, however, there have been few studies investigating whether ME is capable of improving the rate of prediction for pathological diagnosis of gastric scar lesions after ER in early gastric neoplasm beyond that of the conventional method. To the best of our knowledge, this is the first description in the English literature of magnifying endoscopic classification and the characteristic definition of gastric post-ER scar lesions which includes comparative pathology for both magnifying and conventional procedures.

Diagnosis of early gastric cancer relies on macroscopic findings by CE, namely flat, elevated, or depressed; color identical to the neighboring noncancerous area, red or pale; the presence of granules or nodules; the presence or absence of ulcers; and the presence or absence of fold conversions, among others[13]. During diagnostic endoscopy, the endoscopist usually takes routine biopsies from even inconspicuous lesions that appear slightly erythematous, discolored, flat, granular, or shallow depressed mucosal areas in the stomach[14,21]. There has been no definitive description of which findings require endoscopic biopsy or where we have to target the biopsy in post-ER scar lesions in early gastric neoplasm. In our study, nodularity and erythematous lesions revealed the presence of tumors (sensitivity, 88.9% and 100%, respectively). One of 4 ulcer lesions revealed the presence of tumor (sensitivity, 11.1%). These CE findings were important in differentiating tumors in post-ER scars, but these findings in post-ER scar lesions are not specific to tumorous lesions (positive predictive value: 10.6-25.0%) in terms of diagnosing recurrence or suspected tumor in this study. Additionally, these findings give no specific information as to where we must target biopsies in certain large post-ER scar lesions. We cannot ignore endoscopic biopsy in cases with these endoscopic findings, which requires additional costs and is invasive in certain cases with no tumorous post-ER scar lesions.

Nevertheless, there is controversy regarding whether endoscopists should perform a biopsy during every follow-up study after complete ER. Recently, several endoscopists have suggested that short-term endoscopic examination is not necessary since complete ESD was introduced[22]. With recent advances in endoscopic skill and equipment, gastric neoplasms can be resected more completely by ESD, a technique that can produce larger and safer margins around the tumor compared to conventional EMR, thus making the rate of tumor recurrence very low. Recent ESD results have achieved greater than 95% *en bloc* resection as well as excellent survival rates[23,24]. In short-term follow-up endoscopic examinations in post-ER scars, the presence of a tumor can be considered residual tumor rather than the recurrence of a new tumor when we consider the doubling time of early gastric neoplasm.

Using ME, we classified post-ER scar lesions according to the fine gastric mucosal pit patterns of sulci and ridges as follows: (Ⅰ) round pit patterns; (Ⅱ) short rod or tubular pit patterns; (Ⅲ) branched or gyrus-like pit patterns; and (Ⅳ) destroyed pit patterns. Non-tumorous lesions in post-ER scars included type Ⅰ, Ⅱ, and Ⅲ pit patterns, and none of these pit patterns were identified as histologically discernable tumorous lesions

in our study. All the tumor lesions were noted in post-ER scar lesions with the type Ⅳ pattern. Our results suggest that the ME pattern may be considered a useful diagnostic tool capable of replacing the more invasive technique of endoscopic biopsy or identifying the target biopsy site in cases with mixed pit patterns.

Although there was no statistical significance in the relationship between endoscopic findings and other non-neoplastic pathologic findings, type Ⅲ or Ⅳ pit patterns exhibited slightly higher histological scores in terms of gland congestion, intestinal metaplasia, and atrophy. High scores with regard to gland congestion may play a role in the regeneration process, and high scores with regard to the other two findings may be suspected in relation to pathology near the original gastric neoplasm before ER. We were unable to evaluate whether these findings demonstrated a tendency toward tumor development. A longitudinal long-term follow-up study is needed to determine the significance of these non-neoplastic pathologic findings, and a large-scale study is needed to assess the relationship between these pathologic findings and the presence of tumors in post-ER scar lesions.

In this study, none of the patients who had been treated with complete *en bloc* resection by ESD had the type Ⅳ pattern, and no tumor lesions were observed pathologically in these patients. Nine tumor lesions were noted in cases with incomplete resection (6 cases) and piecemeal resection by EMR (3 cases). Consequently, we believe that ME will be useful in predicting the pathological diagnosis of tumorous lesions in post-ER scars, especially after incomplete or piecemeal resection. Furthermore, compared with CE, ME might be a useful alternative to biopsies, especially for short-term follow-up after complete *en bloc* resection by ESD.

There were, however, some limitations to our study: (1) we focused on the simple characteristics of the mucosal pit structures of scar lesions at 2 mo after ER. We could not evaluate the vascular pattern and the validity of various pathologic findings using our short-term results. In addition, we need a long-term follow up study to confirm the final histology in lesions shown to be non-neoplastic in nature with type Ⅰ-Ⅲ pit patterns. (2) We enrolled only nine cases with type Ⅳ pit pattern because the therapeutic outcome of ER is excellent in gastric neoplasms. We could not discuss the diagnostic accuracy overall but could only do so in the nine cases with type Ⅳ pit pattern. A larger study with more cases to obtain a statistically meaningful accuracy is required in order to detect tumor recurrence in scar lesions following ER. (3) In terms of our procedure, the use of a transparent cap limited our survey capacity because it produced a narrow window of view and was very time consuming. After these procedural handicaps are overcome, large-scale and longitudinal follow-up studies should be pursued.

In conclusion, ME findings can detect the presence of tumors through detailed classification of post-ER scar lesions. ME may also help in decision-making regarding whether to perform biopsies and in identifying the target biopsy site in the short-term follow-up of post-ER scars in early gastric neoplasm. As stated above, however, further large-scale and long-term studies are required to determine whether ME can replace endoscopic biopsy.

COMMENTS

Background

Magnifying endoscopy (ME) is now being used in the diagnosis of various gastrointestinal diseases. However, not much data is currently available regarding the correlation between the findings on ME and pathological findings on post-endoscopic resection (ER) scars. There has been no definitive endoscopic description of which endoscopic findings require endoscopic biopsy or where the endoscopist should target the biopsy in altered large scar lesions.

Research frontiers

In this study, the authors demonstrate the relationship between the real-time diagnosis of post-ER scars observed using ME and the pathological diagnosis, thereby validating the clinical usefulness of ME as a follow-up method for post-ER scars in early gastric neoplasm.

Innovations and breakthroughs

This study gives the first description in the English literature of ME classification and the characteristic definition of gastric post-ER scar lesions. In addition, it includes comparative pathology for both the magnifying and conventional findings. Furthermore, our study suggests that ME can detect the presence of tumors through pit classification and may help in decision-making regarding the target biopsies in the short-term follow-up of post-ER scars.

Applications

By providing an understanding of how ME permits visualization of post-ER scars, this study may represent a future strategy in the short-term follow-up of post-ER scars in early gastric neoplasm.

Terminology

The mucosal pits, which were magnified up to 80 times with ME, were classified into four patterns of sulci and ridges: (Ⅰ) round pit patterns; (Ⅱ) short rod or tubular pit patterns; (Ⅲ) branched or gyrus-like pit patterns; and (Ⅳ) destroyed pit patterns. The criteria for suspecting a tumorous lesion included the observation of primarily a destroyed pit pattern.

Peer review

The authors investigated the pit patterns of post-ER scars using ME in early gastric neoplasm. It was revealed that all tumor lesions noted were in the type Ⅳ pit pattern. The results suggest that the ME pit patterns may be considered a useful diagnostic tool capable of replacing the more invasive endoscopic biopsy or of locating the target biopsy site in cases with mixed pit patterns, and may also help in the decision-making regarding whether to perform biopsies in the short-term follow-up of post-ER scars in early gastric neoplasm.

REFERENCES

1 **Kudo S**, Tamura S, Nakajima T, Yamano H, Kusaka H, Watanabe H. Diagnosis of colorectal tumorous lesions by magnifying endoscopy. *Gastrointest Endosc* 1996; **44**: 8-14
2 **Endo T**, Awakawa T, Takahashi H, Arimura Y, Itoh F, Yamashita K, Sasaki S, Yamamoto H, Tang X, Imai K. Classification of Barrett's epithelium by magnifying endoscopy. *Gastrointest Endosc* 2002; **55**: 641-647
3 **Sharma P**. Magnification endoscopy. *Gastrointest Endosc* 2005; **61**: 435-443
4 **Dinis-Ribeiro M**, da Costa-Pereira A, Lopes C, Lara-Santos L, Guilherme M, Moreira-Dias L, Lomba-Viana H, Ribeiro A, Santos C, Soares J, Mesquita N, Silva R, Lomba-Viana R. Magnification chromoendoscopy for the diagnosis of gastric intestinal metaplasia and dysplasia. *Gastrointest Endosc* 2003; **57**: 498-504
5 **Singh R**, Anagnostopoulos GK, Yao K, Karageorgiou H, Fortun PJ, Shonde A, Garsed K, Kaye PV, Hawkey CJ, Ragunath K. Narrow-band imaging with magnification in Barrett's esophagus: validation of a simplified grading system of mucosal morphology patterns against histology.

Endoscopy 2008; **40**: 457-463
6 **Kara MA**, Ennahachi M, Fockens P, ten Kate FJ, Bergman JJ. Detection and classification of the mucosal and vascular patterns (mucosal morphology) in Barrett's esophagus by using narrow band imaging. *Gastrointest Endosc* 2006; **64**: 155-166
7 **Kara MA**, Bergman JJ. Autofluorescence imaging and narrow-band imaging for the detection of early neoplasia in patients with Barrett's esophagus. *Endoscopy* 2006; **38**: 627-631
8 **Kara MA**, Peters FP, Rosmolen WD, Krishnadath KK, ten Kate FJ, Fockens P, Bergman JJ. High-resolution endoscopy plus chromoendoscopy or narrow-band imaging in Barrett's esophagus: a prospective randomized crossover study. *Endoscopy* 2005; **37**: 929-936
9 **Yao K**, Oishi T, Matsui T, Yao T, Iwashita A. Novel magnified endoscopic findings of microvascular architecture in intramucosal gastric cancer. *Gastrointest Endosc* 2002; **56**: 279-284
10 **Yoshida T**, Kawachi H, Sasajima K, Shiokawa A, Kudo SE. The clinical meaning of a nonstructural pattern in early gastric cancer on magnifying endoscopy. *Gastrointest Endosc* 2005; **62**: 48-54
11 **Tamai N**, Kaise M, Nakayoshi T, Katoh M, Sumiyama K, Gohda K, Yamasaki T, Arakawa H, Tajiri H. Clinical and endoscopic characterization of depressed gastric adenoma. *Endoscopy* 2006; **38**: 391-394
12 **Yagi K**, Aruga Y, Nakamura A, Sekine A, Umezu H. The study of dynamic chemical magnifying endoscopy in gastric neoplasia. *Gastrointest Endosc* 2005; **62**: 963-969
13 **Otsuka Y**, Niwa Y, Ohmiya N, Ando N, Ohashi A, Hirooka Y, Goto H. Usefulness of magnifying endoscopy in the diagnosis of early gastric cancer. *Endoscopy* 2004; **36**: 165-169
14 **Tajiri H**, Doi T, Endo H, Nishina T, Terao T, Hyodo I, Matsuda K, Yagi K. Routine endoscopy using a magnifying endoscope for gastric cancer diagnosis. *Endoscopy* 2002; **34**: 772-777
15 **Areia M**, Amaro P, Dinis-Ribeiro M, Cipriano MA, Marinho C, Costa-Pereira A, Lopes C, Moreira-Dias L, Romaozinho JM, Gouveia H, Freitas D, Leitao MC. External validation of a classification for methylene blue magnification chromoendoscopy in premalignant gastric lesions. *Gastrointest Endosc* 2008; **67**: 1011-1018
16 Japanese Gastric Cancer Association. Japanese Classification of Gastric Carcinoma-2nd English Edition. *Gastric Cancer* 1998; **1**: 10-24
17 **Schlemper RJ**, Riddell RH, Kato Y, Borchard F, Cooper HS, Dawsey SM, Dixon MF, Fenoglio-Preiser CM, Flejou JF, Geboes K, Hattori T, Hirota T, Itabashi M, Iwafuchi M, Iwashita A, Kim YI, Kirchner T, Klimpfinger M, Koike M, Lauwers GY, Lewin KJ, Oberhuber G, Offner F, Price AB, Rubio CA, Shimizu M, Shimoda T, Sipponen P, Solcia E, Stolte M, Watanabe H, Yamabe H. The Vienna classification of gastrointestinal epithelial neoplasia. *Gut* 2000; **47**: 251-255
18 **Koba I**, Yoshida S, Fujii T, Hosokawa K, Park SH, Ohtsu A, Oda Y, Muro K, Tajiri H, Hasebe T. Diagnostic findings in endoscopic screening of superficial colorectal neoplasia: results from a prospective study. *Jpn J Clin Oncol* 1998; **28**: 542-545
19 **Yagi K**, Nakamura A, Sekine A. Comparison between magnifying endoscopy and histological, culture and urease test findings from the gastric mucosa of the corpus. *Endoscopy* 2002; **34**: 376-381
20 **Cales P**, Oberti F, Delmotte JS, Basle M, Casa C, Arnaud JP. Gastric mucosal surface in cirrhosis evaluated by magnifying endoscopy and scanning electronic microscopy. *Endoscopy* 2000; **32**: 614-23
21 **Tajiri H**, Ohtsu A, Boku N, Muto M, Chin K, Matsumoto S, Yoshida S. Routine endoscopy using electronic endoscopes for gastric cancer diagnosis: retrospective study of inconsistencies between endoscopic and biopsy diagnoses. *Cancer Detect Prev* 2001; **25**: 166-173
22 **Nakajima T**, Oda I, Gotoda T, Hamanaka H, Eguchi T, Yokoi C, Saito D. Metachronous gastric cancers after endoscopic resection: how effective is annual endoscopic surveillance? *Gastric Cancer* 2006; **9**: 93-98
23 **Lee IL**, Wu CS, Tung SY, Lin PY, Shen CH, Wei KL, Chang TS. Endoscopic submucosal dissection for early gastric cancers: experience from a new endoscopic center in Taiwan. *J Clin Gastroenterol* 2008; **42**: 42-47
24 **Oda I**, Saito D, Tada M, Iishi H, Tanabe S, Oyama T, Doi T, Otani Y, Fujisaki J, Ajioka Y, Hamada T, Inoue H, Gotoda T, Yoshida S. A multicenter retrospective study of endoscopic resection for early gastric cancer. *Gastric Cancer* 2006; **9**: 262-270

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BRIEF ARTICLES

Genomic-wide analysis of lymphatic metastasis-associated genes in human hepatocellular carcinoma

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Abstract

AIM: To identify the genes related to lymph node metastasis in human hepatocellular carcinoma (HCC), 32 HCC patients with or without lymph node metastasis were investigated by high-throughput microarray comprising 886 genes.

METHODS: The samples of cancerous and non-cancerous paired tissue were taken from 32 patients with HCC who underwent hepatectomy with lymph node dissection. Total RNA was extracted from the cells obtained by means of laser microdissection (LCM) and was amplified by the T7-based amplification system. Then, the amplified samples were applied in the cDNA microarray comprising of 886 genes.

RESULTS: The results demonstrated that 25 up-regulated genes such as cell membrane receptor, intracellular signaling and cell adhesion related genes, and 48 down-regulated genes such as intracellular signaling and cell cycle regulator-related genes, were correlated with lymph node metastasis in HCC. Amongst them were included some interesting genes, such as *MET, EPHA2, CCND1, MMP2, MMP13, CASP3, CDH1*, and *PTPN2*. Expression of 16 genes (*MET, CCND1, CCND2, VEGF, KRT18, RFC4, BIRC5, CDC6, MMP2, BCL2A1, CDH1, VIM, PDGFRA, PTPN2, SLC25A5* and *DSP*) were further confirmed by real-time quantitative reverse transcriptional polymerase chain reaction (RT-PCR).

CONCLUSION: Tumor metastasis is an important biological characteristic, which involves multiple genetic changes and cumulation. This genome-wide information contributes to an improved understanding of molecular alterations during lymph node metastasis in HCC. It may help clinicians to predict metastasis of lymph nodes and assist researchers in identifying novel therapeutic targets for metastatic HCC patients.

Key words: Hepatocellular carcinoma; Lymphatic metastasis-associated genes; cDNA microarray; Expression profiling

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INTRODUCTION

Hepatocellular carcinoma (HCC), endemic to sub-Saharan African and Asian with a rising incidence in Western countries, is one of the most common fatal malignancies in the world[1-3]. This is due to different risk factors. Chronic hepatitis B virus (HBV), hepatitis C virus (HCV) infection[4] and exposure to the carcinogen aflatoxin[5] are important risk factors for African and Asian populations. HCC has a poor prognosis, with a 5-year survival of less than 3% in inoperable cases. The high mortality associated with this disease is mainly attributed to its high tendency to metastasize. In fact, local lymph node and blood metastases could occur at an early stage, which may be the key factors related to its recurrence and poor prognosis[5]. Thus, a better understanding of the molecular mechanism of metastasis can improve prevention and treatment of HCC.

Metastatic spread of tumor cells is a process involving multiple steps. To metastasize, tumor cells need to detach from the primary tumor mass, migrate to a distant secondary site, and rapidly expand in the new environment. The whole process requires activation and deactivation of multiple specific genes[6,7]. The present challenge is to identify the crucial genes controlling the metastasis and determine the regulatory mechanism of these genes. Hence, global analysis of expression profiles of a large number of genes in clinical HCC specimens is an essential step to clarify the detailed mechanism and discover potential biomarkers of lymphatic metastasis in HCC. Microarray techniques, which have been developed since the early 1990s, provide a platform where one can measure the expression levels of tens of thousands of genes in a sample simultaneously[8-10], and it is now possible to uncover the complete picture of lymphatic metastasis of HCC. In the current study, we analyzed gene expression profiles in a total of 32 HCC patients using cDNA microarray technology and their relation to pathological features based on lymph node metastasis staging. Validating the cellular functions of these genes will help to identify the key or candidate genes/pathways responsible for lymph node metastasis, which might be used as diagnostic markers and therapeutic targets for lymph node metastasis.

MATERIALS AND METHODS

Patient material

The Institutional Review Board on Medical Ethics, Zhejiang Provincial People Hospital (China), approved the method of tissue collection. The present study was based on 32 patients who underwent hepatectomy for sporadic HCC without preoperative radio- or chemotherapy in the Surgery Department, Zhejiang Provincial People Hospital. All of the samples were immediately frozen in liquid nitrogen, and stored at -80℃ until use. A total of 32 HCC samples from 15 lymph node-negative and 17 lymph node-positive cases were used (Table 1).

Table 1 Clincal data of patients with hepatocellular carcinoma

Case	Sex	Age	Hepatitis virus	Differentiated grade	TNM score
1	M	54	HBV	WD	T1N0M0
2	M	60	HCV	WD	T1N0M0
3	F	61	HBV	WD	T2N0M0
4	M	62	HBV	WD	T2N0M0
5	M	58	HBV	WD	T1N0M0
6	F	56	HCV	MD	T3N0M0
7	F	44	HBV	WD	T2N0M0
8	M	49	HCV	WD	T1N0M0
9	M	58	HBV	WD	T2N0M0
10	M	67	HCV	PD	T3N0M0
11	M	69	HBV	WD	T2N0M0
12	F	63	HCV	WD	T1N0M0
13	M	48	HCV	MD	T2N0M0
14	F	63	HBV	WD	T1N0M0
15	M	49	HCV	MD	T1N0M0
16	F	51	HBV	PD	T3N1M0
17	M	65	HCV	MD	T3N1M0
18	F	58	HBV	PD	T4N1M1
19	M	60	HBV	MD	T2N1M0
20	F	56	HCV	PD	T3N1M1
21	M	42	HCV	PD	T3N1M0
22	M	55	HBV	PD	T4N1M1
23	M	66	HBV	MD	T3N1M0
24	F	70	HCV	WD	T2N1M0
25	M	58	HBV	PD	T4N1M1
26	M	53	HCV	PD	T3N1M0
27	M	61	HBV	PD	T4N1M0
28	F	65	HBV	MD	T3N1M0
29	M	59	HCV	MD	T3N1M1
30	M	50	HBV	PD	T3N1M0
31	F	63	HCV	PD	T4N1M1
32	M	66	HCV	PD	T3N1M0

M: Male; F: Female; HBV: Hepatitis B virus infection; HCV: Hepatitis C virus infection; WD: Well differentiated HCC; MD: Moderately differentiated HCC; PD: Poorly differentiated HCC.

Laser microdissection

The 8 μm-thick sections of frozen tissue were continuously cut at -20℃ and stained with H&E. Under microscopic observation, parts of cancer cell nests in the invasive and intraductal components were microdissected using the LM100 laser capture microdissection system (Arcturus Engineering, Mountain View, CA, USA). We used a 15 μm-diameter beam to capture the tumor cells and corresponding noncancerous liver tissues, respectively. The cell nests were transferred to the laser microdissection (LCM) transfer film (CapSure TF-100S transfer film carrier, 5 mm-diameter optical-grade transparent plastic; Arcturus Engineering).

RNA preparation and T7-based RNA amplification

Total RNA was isolated from the dissected specimens using Trizol reagent (Gibco BRL) and a modified acidic guanidinium phenol-chloroform method, following the manufactures recommendations. Total RNA was treated with DNase I for removal of genomic DNA. mRNA was purified using a poly(A) purification kit (Oligotex, Qiagen) according to the manufactures instructions. The quality of mRNA was assessed by OD 260/280 ratios and the contamination of genomic DNA was checked using the PCR method. cDNA was synthesized with T7-oligo (dT) primer (Ambion) and Superscript Ⅱ enzyme (Gibco BRL) following

the instruction manual. cDNA was purified by cDNA clean-up column (DNA clear™ kit, Ambion). cRNA was generated by T7 MEGAscript™ kit (MEGAscript *in vitro* Transcription Kit, Ambion, Austin, TX, USA) following the manufactures recommendations. Column purification of cRNA was performed with RNeasy kit (Qiagen) according to the manufactures protocol. The concentration and quality of cRNA were analyzed by GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech, Buckinghamshire, England).

Microarray hybridization and scanning

Human Cancer Chip version 4.0 (IntelliGene, TaKaRa) was used for these studies. This array was spotted with 886 cDNA fragments of human genes, which are composed of 588 kinds of human identified genes related to cancer and 298 cDNA fragments prescreened by differential display methods between cancer tissue and normal tissues, on a glass slide. Three μg of cRNA from the tumor and the matched normal tissue were respectively labeled with Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech, Buckinghamshire, England) using a labeling kit (RNA Fluorescence Labeling Core kit, TaKaRa), following the manufactures instructions. Labeled probe was purified by centrifugation in a spin column (Centrisep, Princeton Separations, Adelphia, NJ). Two separate probes were combined, and then, 2 μL of 5 × competitor containing CotI (Gibco BRL), poly dA (Amersham Pharmaca Biotech), and tRNA (TaKaRa) were added. After addition of 50 μL of 100% ethanol and 2 μL of 3 mmol/L sodium acetate (pH 5.2), the mixture was cooled at -80℃ for 30 min, followed by centrifugation at 15000 rpm for 10 min. For final probe preparation, the pellet was washed in 500 μL of 70% ethanol twice, and eluted in 10 μL hybridization buffer (6 × SSC, 0.2% SDS, 5 × Denhardt's solution, 0.1 mg/mL salmon sperm solution). The probes were denatured by heating for 2 min at 95℃, cooled at room temperature, and centrifuged at 15000 rpm for 10 min (20-26℃). Supernatants were placed on the array and covered with a 22 mm × 22 mm glass coverslip. The coverslip was sealed with a glue, and the probes were incubated overnight at 65℃ for 16 h in a custom-made slide chamber with humidity maintained by underlying moist papers. After hybridization, the slides were washed in 2 × SSC with 0.1% SDS, 1 × SSC, and 0.05 × SSC, sequentially for 1 min each, and then spin dried. Hybridized arrays were scanned using a confocal laser-scanning microscope (Affymetrix 428 array scanner, Santa Clara, CA). Image analysis and quantification were performed with ImaGene 4.2 software (BioDiscovery) as per the manufactures instructions.

Data processing

Each spot was defined by manual positioning of a grid of circles over the array image. For each fluorescent image, the average pixel intensity within each circle was determined, and a local background outside of 3 pixel buffer range from the circle was computed for each spot. Net signal intensity was determined by subtraction of this local background from the average intensity of each spot. Signal intensities between the two fluorescent images were normalized by the intensities of the housekeeping genes provided on the arrays. The fluorescence intensities of Cy5 (non-tumor) and Cy3 (tumor) for each target spot were adjusted so that the mean Cy3:Cy5 ratios of 32 housekeeping gene spots were equal to one. Because data derived from low signal intensities are less reliable, we first determined cutoff values for signal intensities on each slide so that all of the filtered genes had greater S:N (signal to noise) ratios of Cy3 or Cy5 than three, and we excluded genes for further analysis when both Cy3 and Cy5 dyes gave signal intensities lower than the cutoff. To estimate the range of expression ratio within which the expression change could be considered as fluctuation in noncancerous cells, we compared expression profiles of noncancerous cells from 6 patients. Because 90% of expression ratios in noncancerous cells fell within the range of 1.726 and 0.503, we categorized genes into three groups according to their expression ratios (Cy3:Cy5): up-regulated (ratio: 2.0); down-regulated (ratio: 0.5); and unchanged expression (ratios between 0.5 and 2.0); provided that signal counts of T (Cy3) and R (Cy5) were > 500. Genes with Cy3:Cy5 ratios > 2.0 or < 0.5 in more than 75% of the cases examined were defined as commonly up- or down-regulated genes, respectively.

Real-time reverse transcription-PCR

LightCycler (Roche Diagnostics) technology was applied to confirm the data which were obtained by cDNA microarray. The primer sequences of 16 genes were obtained from the GDB Human Genome Database (http://www.gdb.org/gdb/). We used the same RNA from the dissected cells in microarray analysis. First-strand cDNA was obtained by reverse transcription using a commercially available kit (first strand synthesis kit, Amersham). For each PCR, 2 μL (20 ng) first strand cDNA template, 50 pmol of each primer, 2.4 μL (3 mmol/L) $MgCl_2$, and 2 μL 10 × SYBR Green I (Roche Laboratories) were mixed in 20 μL of PCR mixture. The running protocol has been programmed based on the following three steps. In the first step, initial denaturation, reaction mixture was incubated for 10 min at 95℃. In the second step, DNA was amplified for 45 cycles at 95℃ for 10 s, specific annealing temperature (the primer sequences dependent) for 0-10 s, and elongation at 72℃ for some seconds (amplicon [bp]/25 s). Finally, the temperature was raised gradually (0.2℃/s) from the annealing temperature to 95℃ for the melting curve analysis. Twelve μL of PCR products were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide. The amount of gene expression was normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Human GAPDH kit (GmbH Heidelberg, Heidelberg, Germany). We carried out qRT-PCR analysis in triplicate for each cDNA sample and used median values in three experiments. Up- and down-regulation were defined as the median value > 2.0 and < 0.5, respectively.

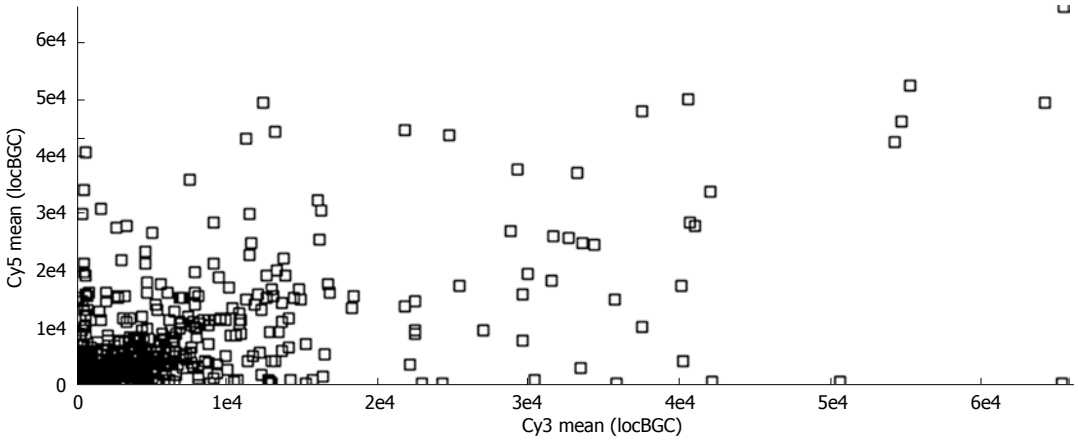

Figure 1 Scatter plots of cDNA microarray analysis. Primary carcinoma cells (Cy3-labeled) and normal cells (Cy5-labeled) from case 20 are labeled and hybridized to the cDNA microarray.

Statistical analysis

A statistical analysis among mean values was performed on the association of lymph node metastasis with expression levels by applying non-parametric Kruskal-Wallis and Mann-Whitney *U* tests. Statistical significance was defined as a *P*-value < 0.05. Differential expression between the groups of lymph node metastasis and the group of non-lymph metastasis was considered significant, where *P* value < 0.05.

RESULTS

Quality analysis of total RNA after LCM and cRNA after T7-based amplification was carried out. About 20 slides were prepared in every sample, and the target cells were captured with at least approximately 1000 cells per slide. Consequently, we captured a total of approximately 25000-30000 tumor and normal cells for RNA extractions, respectively. The quality of total RNA extracted after LCM was assessed by A260/A280 and electrophoresis. To be considered for microarray analysis, the RNA samples needed to pass the quality control criteria, namely integrity of 28S and 18S, and A260/A280 greater than 2.0. Products of cDNA synthesis and cRNA were also checked by A260/A280 and electrophoresis. Results showed that A260/A280 of all the RNA samples met the quality control criteria for sample preparation. Clear image appearance of 28S and 18S of ribosomal RNA was seen under the electropherogram for each total RNA sample, which had to be considered as intact or without degradation. RNA was subjected to two rounds of T7-based RNA amplification after removal of DNA contamination by RNase-free DNase I treatment as described in Methods. All RNA was successfully amplified an estimated 250-fold by using T7 RNA polymerase. cDNA synthesis and cRNA showed satisfactory quality control criteria, which was 1.5 kb < cDNA < 5.0 kb; 1.0 kb < cRNA < 4.5 kb); and A260/A280 ratio of cDNA and cRNA greater than 2, respectively.

Identification of expressed genes associated with lymph node metastasis

After reverse transcription, each cDNA probe was labeled with Cy3- or Cy5-conjugated dyes and hybridized to microarray cDNAs with 886 genes. We evaluated the expression profiles comparing the cancer cells and the corresponding normal cells in each case. A representative scatter plot of microarray analysis between the metastatic carcinoma cells and non-cancerous tissue in case 20 is shown in Figure 1. Up-, down-regulated and unchanged genes indicated by red, green and blue spots respectively are shown in Figure 2. We first arranged the relative expression of each gene (Cy3/Cy5 intensity ratio) into one of four categories: up-regulated (ratio: > 2.0), down-regulated (ratio: < 0.5), unchanged (ratio: between 0.5 and 2.0), and not expressed (or slight expression but under the cutoff level for detection).

To identify the genes related to lymph node metastasis, 32 cases were divided into two groups: a metastatic group in which lymph node metastasis was positive in 17 patients (No. 16-32) and a non-metastatic group in which lymph node metastasis was negative in 15 patients (No. 1-15) (Table 1). When comparing gene expression profiles between two groups, there were 25 genes that were commonly up-regulated and expressed more than 1.87-fold in the lymphatic metastasis groups compared with those in the negative groups. On the other hand, 48 down-regulated expressed genes were significantly correlated with the lymphatic metastasis groups. Tables 2 and 3 show the list of these differentially expressed genes and their category based on GO (Gene Ontology) system and TreeView. The up-regulated genes were associated with cell adhesion molecules, cell membrane receptors, intracellular signaling related genes, etc. The up-regulated genes included interesting genes, such as *MET*, *EPHA2*, *CCND1*, *MMP2* and *MMP13*. The down-regulated genes were mostly cell adhesion molecules, cell cycle regulators and intracellular signaling molecules. The down-regulated genes included *CASP3*, *CDH1*, and *PTPN2*.

Gene expression confirmation by real-time RT-PCR

To investigate the reliability of cDNA microarray data, real-time quantitative RT-PCR was performed for measuring the expression levels of 16 genes (*MET*, *CCND1*, *CCND2*, *VEGF*, *KRT18*, *RFC4*, *BIRC5*, *CDC6*,

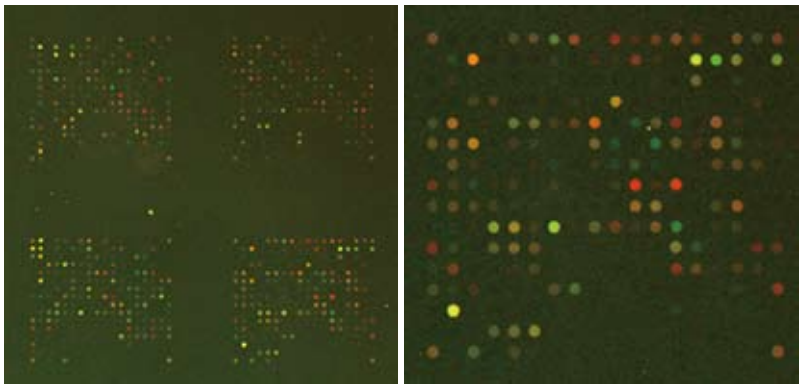

Figure 2 A representative of cDNA microarray expression pattern obtained from case 20. Up-, down-regulated and unchanged genes are indicated by red, green and blue spots, respectively.

Table 2 Up-regulated genes correlated with lymphatic metastasis

Gene name	Symbol[a]	Accession[b]	Fold change[c]	pN1:pN0[d]
Cell adhesion proteins				
CD58 antigen, (lymphocyte function-assocoated antigens)	CD58	NM_001779	7.75	2.28
Integrin αM	ITGAM	NM_000632	5.04	2.06
Integrin β5	ITGB5	NM_002213	3.98	1.87
Opioid-binding protein/cell adhesion molecule-like	OPCML	NM_002545		
Cell membrane receptor				
CD86 antigen, (CD28 antigen ligand 2, B7-2 antigen)	CD86	NM_006889	6.76	2.33
v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	NM_002228	7.43	2.27
Met proto-oncogene (hepatocyte growth factor receptor)	MET	NM_000245	10.11	3.46
EphA2	EPHA2	NM_004431	8.26	2.13
Epidermal growth factor receptor [avian erythroblastic leukemia viral (v-erb-b) oncogene homolog]	EGFR	NM_005228	8.35	2.62
Cell death regulator				
BCL2/adenovirus E1B 19kDa interacing protein 3	BNIP3	NM_004052	3.88	2.29
Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	SEMA4D	NM_006378	5.71	3.64
Intracellular signaling				
Rho GDP dissociation inhibitor γ	ARHGDIG	NM_001175	5.37	1.97
Ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	RAC3	AK054993	4.63	2.07
Insulin-like growth factor binding protein 3	IGFBP3	M35878	5.39	2.15
Coagulation factor Ⅱ (thrombin) receptor	F2R	NM_001992	6.17	2.43
Growth/differentiation factor				
Vascular endothelial growth factor C	VEGFC	NM_005429	5.68	2.47
Cell cycle regulator				
Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	NM_053056	11.56	4.31
Cyclin-dependent kinase 4	CDK4	NM_000075	8.73	3.59
Others				
Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	TIMP3	NM_000362	3.95	1.89
Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	UBE2A	NM_003336	4.38	1.95
v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	YES1	NM_005433	5.66	2.37
P450(cytochrome) oxidoreductase	POR	AF258341	4.74	1.99
Matrix metalloproteinase 13	MMP13	NM_00247	5.67	2.45
Matrix metalloproteinase 2 (gelatinase A, 72kD gelatinase, 72kD type Ⅳ collagenase)	MMP2	NM_004530	7.13	3.36

[a]Symbol in LocusLink database; [b]GeneBank accession number; [c]Fold change, ratio of mean expression values in lymph node metastasis cases (cancer cells *vs* non-cancerous cells); [d]pN1:pN0, ratio of mean expression values (lymph node positive cases to lymph node negative cases).

MMP2, *BCL2A1*, *CDH1*, *VIM*, *PDGFRA*, *PTPN2*, *SLC25A5* and *DSP*). One representative case (case 8) is shown in Figure 3. We used cDNA synthesized from 32 pair samples without amplification as template for real-time quantitative reverse transcription PCR. The results demonstrated that the samples obtained by means of T7-based amplification well reflected the status of the original RNA in a proportional manner.

DISCUSSION

The application of high-throughput cDNA microarray permits simultaneous analysis of genome-wide expression of thousands of genes in a sample and to investigate the correlation between clinicopathological phenotypes and gene expression status[8-10]. This technology is a powerful tool for screening genes,

Table 3 Down-regulated genes correlated with lymphatic metastasis

Gene name	Symbol[a]	Accession[b]	Fold change[c]	pN1:pN0[d]
Cell adhesion proteins				
Desmoplakin (DPI, DPII)	DSP	NM_004415	-14.22	0.24
Protocadherin gamma subfamily C, 3	PCDHGC3	NM_002588	-7.88	0.41
Integrin, beta 4	ITGB4	NM_000213	-13.39	0.26
Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	ITGA3	NM_002204	-6.24	0.38
Catenin (cadherin-associated protein), alpha 1 (102kD)	CTNNA1	NM_001903	-8.53	0.37
Cadherin 1, type 1, E-cadherin (epithelial)	CDH1	NM_004360	-15.85	0.25
Cell cycle regulator				
Cell division cycle 25B	CDC25B	NM_021784	-6.90	0.46
Cyclin-dependent kinase 5	CDK5	NM_004935	-5.51	0.48
Cyclin D2	CCND2	NM_001759	-6.29	0.43
Microtubule-associated protein, RP/EB family, member 1	MAPRE1	NM_012325	-13.16	0.29
Protein phosphatase 1D	PPM1D	NM_003620	-11.77	0.39
Ataxia telangiectasia and Rad3 related	ATR	NM_001184	-7.86	0.41
Protein phosphatase 2, regulatory subunit B (B56), alpha isoform	PPP2R5A	NM_006243	-3.89	0.49
Intracellular signaling				
Mitogen-activated protein kinase 14	MAPK14	NM_001315	-5.67	0.43
Mitogen-activated protein kinase 7	MAPK7	NM_002749	-7.15	0.39
Mitogen-activated protein kinase 4	MAPK4	NM_002747	-3.83	0.32
Small inducible cytokine B subfamily (Cys-X-Cys motif), member 13 (B-cell chemoattractant)	SCYB13	NM_006419	-4.58	0.46
Signal transducer and activator of transcription 5B	STAT5B	NM_012448	-8.13	0.37
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	SERPINB1	NM_030666	-4.47	0.48
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	SERPINB2	NM_002575	-5.72	0.41
SH3-domain binding protein 2	SH3BP2	AB000462	-3.29	0.52
G protein-coupled receptor kinase 6	GPRK6	NM_002082	-14.12	0.29
GTP-binding protein ragB	RAGB	NM_016656	-6.77	0.46
Protein tyrosine phosphatase, receptor type, F	PTPRF	NM_002840	-7.21	0.34
Cell membrane receptor				
EphB6	EPHB6	NM_004445	-12.88	0.38
Kangai 1 (suppression of tumorigenicity 6, prostate; CD82 antigen (R2 leukocyte antigen, antigen detected by monoclonal and antibody IA4)	KAI1	NM_002231	-2.24	0.67
Small inducible cytokine subfamily A (Cys-Cys), member 25	SCYA25	NM_005624	-3.76	0.72
Monoglyceride lipase	MGLL	NM_007283	-4.66	0.43
Interferon (alpha, beta and omega) receptor 1	IFNAR1	NM_000629	-2.39	0.81
Insulin-like growth factor binding protein 6	IGFBP6	NM_002178	-3.53	0.62
Metabolic enzyme				
Serine protease inhibitor, Kunitz type, 2	SPINT2	NM_021102	-5.44	0.46
Protein phosphatase 2, regulatory subunit B (B56), alpha isoform	PPP2R5A	NM_006243	-7.12	0.37
Protein tyrosine phosphatase, non-receptor type 2	PTPN2	NM_002828	-19.73	0.23
Deoxyribonuclease I-like 3	DNASE1L3	NM_004944	-8.23	0.29
Cathepsin L	CTSL	NM_001912	-4.39	0.37
Cell death regulator				
Caspase 3, apoptosis-related cysteine protease	CASP3	NM_004346	-14.57	0.29
Programmed cell death 10	PDCD10	NM_007217	-7.19	0.36
DNA damage response				
Ataxia telangiectasia and Rad3 related	ATR	NM_001184	-4.78	0.48
X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80kD)	XRCC5	NM_021141	-2.59	0.73
Mouse double min 2	MDM2	NM_002392	-6.37	0.51
Growth/differentiation factor				
Bone morphogenetic protein 5	BMP5	NM_021073	-2.78	0.53
Keratin 4	KRT4	NM_002272	-3.12	0.62
Keratin 13	KRT13	NM_002274	-4.24	0.48
Connective tissue growth factor	CTGF	NM_001901	-5.51	0.42
Others				
Heat shock 70kDa protein 4	HSPA4	AB023420	-3.19	0.56
Retinoid X receptor, α	RXRA	NM_002957	-2.89	0.77
Ubiquitin-activating enzyme E1-like	UBE1L	NM_003335	-2.95	0.53
Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	SLC25A5	NM_001152	-6.18	0.46

[a]Symbol in LocusLink database; [b]GeneBank accession number; [c]Fold change, ratio of mean expression values in lymph node metastasis cases (cancer cells *vs* non-cancerous cells); [d]pN1:pN0, ratio of mean expression values (lymph node positive cases to lymph node negative cases).

the expression of which can be correlated with pathological phenotypes of various tumors[11,12]. Analysis of gene expression profiles not only has disclosed specific patterns that may reflect prognosis and drug sensitivity of tumor cells but has also revealed the identity of genes involved in malignant transformation,

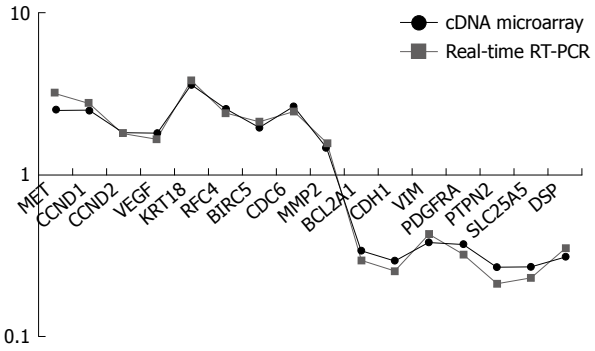

Figure 3 Relative abundance of the mRNA levels of 16 genes in one representative case (case 8), determined by cDNA microarray and real-time RT-PCR, respectively.

progression, and metastasis of tumors[13-16]. However, the existence of bulky surrounding cells produces much interferential noise information because of interstitial effects[17]. Therefore, selection of cancer cells using LCM is of indispensable value in combination with the cDNA microarray. This study clearly demonstrated that an analysis of global gene expression profiles can be performed with RNA samples obtained from primary HCC tissues by using LCM, T7-based RNA amplification, and cDNA microarray. It is thus possible to focus directly on the gene expression profile of an individual cell population consisting of tumor tissue.

Lymph node metastasis is one of the most important prognostic factors in HCC patients[18,19]. This is a highly selective sequential step involving multiple genes, multiple signals pathways and regulatory mechanisms during the process, which favors the survival of a subpopulation of metastatic cells preexisting within the primary tumor mass to produce clinically relevant metastases[20-23]. The metastatic cells exhibit a complex phenotype that is regulated by transient or permanent alterations in various genes at mRNA level. In the present study of HCC patients, we compared gene expression in lymph node metastasis and in those without lymph node metastasis by using cDNA microarray analysis. We found that 25 up-regulated and 48 down-regulated genes were correlated with lymph node metastasis of HCC. The up-regulated genes included cell adhesion related genes (*CD58*, *ITGAM*, *ITGB5*, *OPCML*), cell membrane receptor (*CD86*, *JUN*, *MET*, *EPHA2*, *EGFR*), cell death regulator (*BNIP3*, *SEMA4D*), intracellular signaling related genes (*ARHGDIG*, *RAC3*, *IGFBP3*, *F2R*), growth/differentiation factor (*VEGFC*), cell cycle regulator (*CCND1*, *CDK4*), and other genes (*TIMP3*, *UBE2A*, *YES1*, *POR*, *MMP13*, *MMP2*). The down-regulated genes included cell adhesion related genes (*DSP*, *PCDHGC3*, *ITGB4*, *ITGA3*, *CTNNA1*, *CDH1*), cell cycle regulator (*CDC25B*, *CDK5*, *CCND2*, *MAPRE1*, *PPM1D*, *ATR*, *PPP2R5A*), intracellular signaling related genes (*MAPK14*, *MAPK7*, *MAPK4*, *SCYB13*, *STAT5B*, *SERPINB1*, *SERPINB2*, *SH3BP2*, *GPRK6*, *RAGB*, *PTPRF*), cell membrane receptor (*EPHB6*, *KAI1*, *SCYA25*, *MGLL*, *IFNAR1*, *IGFBP6*), metabolic enzyme (*SPINT2*, *PPP2R5A*, *PTPN2*, *DNASE1L3*, *CTSL*), cell death regulator (*CASP3*, *PDCD10*), DNA damage response related genes (*ATR*, *XRCC5*, *MDM2*), growth/differentiation factor (*BMP5*, *KRT4*, *KRT13*, *CTGF*), and other genes (*HSPA4*, *RXRA*, *UBE1L*, *SLC25A5*).

Amongst these genes are some which are well documented in the literature as being involved in the malignant potential of some types of carcinomas. CCND1, one of the cell cycle regulators, is synthesized in the G1 phase, mixed with CDK2, CDK4 and CDK5, and related closely with cell cycle control[24,25]. Our results showed that CCND1 is highly expressed in HCC with lymphatic metastasis, which may be a useful marker that relates with the poor prognosis of HCC, and overexpression of CCND1 in HCC without lymphatic metastasis may be used as useful information for treatment measures after operation[26,27]. CDK4, an important member of the CDKs protein family, was correlated positively with CCND1 as reported by some documents[24-26]. Our results showed that CDK4 was remarkably up-regulated in HCC patients with lymph node metastasis, and was consistently correlated with that of CCND1[28]. EphA2, one member of the tyrosine activating enzyme acceptor Eph family, can be used as a ligand of ephrins connecting with cells and involved in cell interaction[29]. Our study demonstrated that up-regulation of EphA2 was related to lymph node metastasis of HCC, however, the mechanism remains in need of further studies. It has been proven that the formation of new blood vessels plays a role in the growth and metastasis of solid tumors, and various growth factors secreted from tumor cells determine the pace of the progression process[30]. It is documented that VEGF is up-regulated in most solid tumors, whereas there is few or none in the normal tissues, and its expression level is positively related with microvessel density, invasion, metastasis and prognosis of tumors[31]. Present results showed that the expression levels of VEGF were related closely to the clinical stage of HCC, its expression levels increased gradually with the increase of TNM, there were significant differences in expression levels between HCC patients with and those without lymph node metastasis ($P < 0.05$), and its expression levels were a useful marker of the recurrence, the distant metastasis and the poor prognosis of HCC (data not shown). The extracellular matrix (ECM) is the first barrier during the process of invasion and metastasis of tumor cells[32]. Tumor cells and their neighbor interstitial cells, such as the endothelial cell, the macrophage and so on, may produce a great quantity of proteinase for degradation of ECM, and help tumor cells to migrate easily. Of these proteinases, the matrix metalloproteinase (MMP) is an important one. MMPs can degrade the stroma collage, which favors the action of tumor cells to shake off the yoke of ECM and to migrate. This is the biochemical basis of the circumambience invasion of tumor cells[33,34]. It was found that MMP13 and MMP2 expressions in HCC with lymph node metastasis were significantly higher than that of those without lymph node metastasis, and MMP2 in the lymph

node metastasis group was 3.12-fold of those without metastasis. Lymph node metastasis of HCC may be related to the "thundering" activity of the growth factor signaling pathway[35]. It has been proven that the signaling pathway of tyrosine kinase receptor and G-protein connective receptor are the most important two of all pathways[36]. EGFR, containing the sequence of tyrosine kinase, was always up-regulated in HCC with lymph node metatasis patients. It has been reported that the signaling pathway mediated by all growth factors for cell proliferation purposes was always dependent on the tyrosine kinase receptor signaling pathway, and tyrosine kinase was kept activated in many tumors[37].

It has been reported that down-regulation of desmoplakin (DSP, member of the cadherin family) is correlated with tumor invasion and metastasis[38]. Our results showed that DSP was suppressed in the lymph node metastasis group compared with the non-metastasis group. Tumor cells always secrete some new adhesion molecules when removed from the primary lesion and grown again in another position, whereas CTNNA 1 was down-regulated in our results, suggesting its role was maybe different from that of the primary lesion[39,40]. E-cadherin (a cell membrane superficial molecule mediating adhesion among normal cells) was down-regulated in lymph node metastasis HCC patients. Ephrin, ligand of tyrosine kinase receptor, was also down-regulated in the metastasis group. It was reported that the combination of ephrin-B2 ligand and EphB4 was related to the occurrence and metastasis of some solid tumors[41,42]. KAI1, a new gene found recently, was down-regulated in many lymphatic metastasis tumors, and this gene is associated with the motion and metastasis of tumor cells[43]. Protein tyrosine phosphatase, which has an action contrary to that of tyrosine kinase, takes part in signal regulation, energy metabolism, cell proliferation and promoting MHCI expression mediated by many hormones, such as insulin and epidermal growth factor, and others[44,45]. The decrease of tyrosine phosphatase activity may reduce the MHCI expression of cells superficially so that tumor cells escape the inspection of the immunological system[44]. PTPN2 and PTPRF were down-regulated in the lymph node metastasis group, suggesting that the tyrosine phosphatase was associated with invasion and metastasis of tumor cells[44-46].

Our study demonstrated that lymph node metastasis comes from the result of the structural and functional abnormality of cellular and extracellular multigenes, since many genes and signaling pathways play key roles during the metastasis of tumor cells by dominating the cell proliferation, differentiation and death. Potential metastatic biological behavior of tumor cells was characteristically the release of cell-cell adhesion, the abnormality of cell cycle regulator and cell signal pathways, which suggest that the invasive character of tumor cells is determined by cellular interaction with the extracellular environment rather than the proliferative potential. The abnormality of apoptosis and proliferation ability may occur owing to loss of control of the cell cycle and the obstruction of cell signal molecules transmitting communication, which may be is one of the mechanisms of accelerating tumor invasion and metastasis[47,48]. Although we were able to extract some genes related to lymph node metastasis in HCC, further examination is necessary of other genes as well as the interaction with stromal tissues. Since these genes are thought to affect each other, it is important to further analyze each gene in detail to elucidate the mechanism of lymph node metastasis in HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC), endemic to sub-Saharan Africa and Asia with a rising incidence in Western countries, is one of the most common fatal malignancies in the world. The high mortality associated with this disease is mainly attributed to its high tendency to metastasize. In fact, local lymph node and blood metastases could occur at an early stage, which may be the key factors relating to its recurrence and poor prognosis. Thus, a better understanding of the molecular mechanism of metastasis can improve prevention and treatment of HCC.

Research frontiers

The purpose of this study is to identify the genes related to lymph node metastasis in human hepatocellular carcinoma (HCC). Thirty two HCC patients with or without lymph node metastases were investigated by high-throughput microarray comprising 886 genes. The results demonstrated that 25 up-regulated genes such as cell membrane receptor, intracellular signaling and cell adhesion related genes, and 48 down-regulated genes such as intracellular signaling and cell cycle regulator-related genes, were correlated with lymph node metastasis in HCC.

Innovations and breakthroughs

The application of high-throughput cDNA microarray permits analysis of genome-wide expression of thousands of genes in a sample and thus to investigate the correlation between clinicopathological phenotypes and gene expression status. This study clearly demonstrated that an analysis of global gene expression profiles can be performed with RNA samples obtained from primary HCC tissues by using LCM, T7-based RNA amplification, and cDNA microarray. Thus, it is possible to focus directly on the gene expression profile of an individual cell population consisting of tumor tissue.

Applications

This genome-wide information contributes to an improved understanding of molecular alterations during lymph node metastasis in HCC. It may help clinicians to predict metastasis of lymph nodes and assist researchers in identifying novel therapeutic targets for metastatic HCC patients.

Terminology

DNA microarray is a high-throughput and powerful technology used in molecular biology and in biomedicine areas. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides. A short section of a gene or other DNA element can be used as a probe to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Peer review

The authors claimed that the expression of distinct sets of genes was either enhanced or decreased in hepatocellular carcinoma tissues with lymph node metastasis, compared with those without any lymph node metastasis. Based on the clinical information described in this article, the patients with lymph node metastasis are in more advanced stages (T3, T4, or M1), compared with those without metastasis. Thus, their observed changes in gene expression may arise from the local tumor growth or distant metastasis. The authors should exclude this possibility.

REFERENCES

1 **Taylor-Robinson SD**, Foster GR, Arora S, Hargreaves S,

Thomas HC. Increase in primary liver cancer in the UK, 1979-94. *Lancet* 1997; **350**: 1142-1143
2 **Deuffic S**, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. *Lancet* 1998; **351**: 214-215
3 **El-Serag HB**, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**: 745-750
4 **Beasley RP**, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. *Lancet* 1981; **2**: 1129-1133
5 **Kasai Y**, Takeda S, Takagi H. Pathogenesis of hepatocellular carcinoma: a review from the viewpoint of molecular analysis. *Semin Surg Oncol* 1996; **12**: 155-159
6 **Wu Y**, Zhou BP. New insights of epithelial-mesenchymal transition in cancer metastasis. *Acta Biochim Biophys Sin* (Shanghai) 2008; **40**: 643-650
7 **Turner DP**, Findlay VJ, Kirven AD, Moussa O, Watson DK. Global gene expression analysis identifies PDEF transcriptional networks regulating cell migration during cancer progression. *Mol Biol Cell* 2008; **19**: 3745-3757
8 **Waddell N**. Microarray-based DNA profiling to study genomic aberrations. *IUBMB Life* 2008; **60**: 437-440
9 **Kuehn H**, Liberzon A, Reich M, Mesirov JP. Using GenePattern for gene expression analysis. *Curr Protoc Bioinformatics* 2008; Chapter **7**: Unit 7.12
10 **Grant SF**, Hakonarson H. Microarray technology and applications in the arena of genome-wide association. *Clin Chem* 2008; **54**: 1116-1124
11 **Yan B**, Chen G, Saigal K, Yang X, Jensen ST, Van Waes C, Stoeckert CJ, Chen Z. Systems biology-defined NF-kappaB regulons, interacting signal pathways and networks are implicated in the malignant phenotype of head and neck cancer cell lines differing in p53 status. *Genome Biol* 2008; **9**: R53
12 **Sabates-Bellver J**, Van der Flier LG, de Palo M, Cattaneo E, Maake C, Rehrauer H, Laczko E, Kurowski MA, Bujnicki JM, Menigatti M, Luz J, Ranalli TV, Gomes V, Pastorelli A, Faggiani R, Anti M, Jiricny J, Clevers H, Marra G. Transcriptome profile of human colorectal adenomas. *Mol Cancer Res* 2007; **5**: 1263-1275
13 **Floris G**, Debiec-Rychter M, Wozniak A, Magrini E, Manfioletti G, De Wever I, Tallini G, Sciot R. Malignant ectomesenchymoma: genetic profile reflects rhabdomyosarcomatous differentiation. *Diagn Mol Pathol* 2007; **16**: 243-248
14 **Mishra PJ**, Mishra PJ, Humeniuk R, Medina DJ, Alexe G, Mesirov JP, Ganesan S, Glod JW, Banerjee D. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. *Cancer Res* 2008; **68**: 4331-4339
15 **Riker AI**, Enkemann SA, Fodstad O, Liu S, Ren S, Morris C, Xi Y, Howell P, Metge B, Samant RS, Shevde LA, Li W, Eschrich S, Daud A, Ju J, Matta J. The gene expression profiles of primary and metastatic melanoma yields a transition point of tumor progression and metastasis. *BMC Med Genomics* 2008; **1**: 13
16 **Rizki A**, Weaver VM, Lee SY, Rozenberg GI, Chin K, Myers CA, Bascom JL, Mott JD, Semeiks JR, Grate LR, Mian IS, Borowsky AD, Jensen RA, Idowu MO, Chen F, Chen DJ, Petersen OW, Gray JW, Bissell MJ. A human breast cell model of preinvasive to invasive transition. *Cancer Res* 2008; **68**: 1378-1387
17 **Gruber HE**, Mougeot JL, Hoelscher G, Ingram JA, Hanley EN Jr. Microarray analysis of laser capture microdissected-anulus cells from the human intervertebral disc. *Spine* 2007; **32**: 1181-1187
18 **Zhuang PY**, Zhang JB, Zhu XD, Zhang W, Wu WZ, Tan YS, Hou J, Tang ZY, Qin LX, Sun HC. Two pathologic types of hepatocellular carcinoma with lymph node metastasis with distinct prognosis on the basis of CK19 expression in tumor. *Cancer* 2008; **112**: 2740-2748
19 **Li Y**, Tian B, Yang J, Zhao L, Wu X, Ye SL, Liu YK, Tang ZY. Stepwise metastatic human hepatocellular carcinoma cell model system with multiple metastatic potentials established through consecutive in vivo selection and studies on metastatic characteristics. *J Cancer Res Clin Oncol* 2004; **130**: 460-468
20 **Kovacevic Z**, Richardson DR. The metastasis suppressor, Ndrg-1: a new ally in the fight against cancer. *Carcinogenesis* 2006; **27**: 2355-2366
21 **Liu S**, Yu M, He Y, Xiao L, Wang F, Song C, Sun S, Ling C, Xu Z. Melittin prevents liver cancer cell metastasis through inhibition of the Rac1-dependent pathway. *Hepatology* 2008; **47**: 1964-1973
22 **Aishima S**, Nishihara Y, Iguchi T, Taguchi K, Taketomi A, Maehara Y, Tsuneyoshi M. Lymphatic spread is related to VEGF-C expression and D2-40-positive myofibroblasts in intrahepatic cholangiocarcinoma. *Mod Pathol* 2008; **21**: 256-264
23 **Gu T**, Kilinc MO, Egilmez NK. Transient activation of tumor-associated T-effector/memory cells promotes tumor eradication via NK-cell recruitment: minimal role for long-term T-cell immunity in cure of metastatic disease. *Cancer Immunol Immunother* 2008; **57**: 997-1005
24 **Sanchez G**, Delattre O, Auboeuf D, Dutertre M. Coupled alteration of transcription and splicing by a single oncogene: boosting the effect on cyclin D1 activity. *Cell Cycle* 2008; **7**: 2299-2305
25 **Kobel M**, Huntsman D, Gilks CB. Critical molecular abnormalities in high-grade serous carcinoma of the ovary. *Expert Rev Mol Med* 2008; **10**: e22
26 **Zhao P**, Lu Y, Zhong M, Liu L, Li B. Inverse correlation of aberrant expression of fragile histidine triad (FHIT) protein with cyclin D1 protein and prognosis in Chinese patients with cholangiocarcinoma. *Acta Oncol* 2008; **47**: 1557-1563
27 **Strong VE**, Kennedy T, Al-Ahmadie H, Tang L, Coleman J, Fong Y, Brennan M, Ghossein RA. Prognostic indicators of malignancy in adrenal pheochromocytomas: clinical, histopathologic, and cell cycle/apoptosis gene expression analysis. *Surgery* 2008; **143**: 759-768
28 **Kishimoto I**, Mitomi H, Ohkura Y, Kanazawa H, Fukui N, Watanabe M. Abnormal expression of p16(INK4a), cyclin D1, cyclin-dependent kinase 4 and retinoblastoma protein in gastric carcinomas. *J Surg Oncol* 2008; **98**: 60-66
29 **Mudali SV**, Fu B, Lakkur SS, Luo M, Embuscado EE, Iacobuzio-Donahue CA. Patterns of EphA2 protein expression in primary and metastatic pancreatic carcinoma and correlation with genetic status. *Clin Exp Metastasis* 2006; **23**: 357-365
30 **Hirakawa S**, Hong YK, Harvey N, Schacht V, Matsuda K, Libermann T, Detmar M. Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am J Pathol* 2003; **162**: 575-586
31 **Qu Z**, Van Ginkel S, Roy AM, Westbrook L, Nasrin M, Maxuitenko Y, Frost AR, Carey D, Wang W, Li R, Grizzle WE, Thottassery JV, Kern FG. Vascular endothelial growth factor reduces tamoxifen efficacy and promotes metastatic colonization and desmoplasia in breast tumors. *Cancer Res* 2008; **68**: 6232-6240
32 **Lunt SJ**, Chaudary N, Hill RP. The tumor microenvironment and metastatic disease. *Clin Exp Metastasis* 2008 [Epub ahead of print]
33 **Rydlova M**, Holubec L Jr, Ludvikova M Jr, Kalfert D, Franekova J, Povysil C, Ludvikova M. Biological activity and clinical implications of the matrix metalloproteinases. *Anticancer Res* 2008; **28**: 1389-1397
34 **Oshima T**, Kunisaki C, Yoshihara K, Yamada R, Yamamoto N, Sato T, Makino H, Yamagishi S, Nagano Y, Fujii S, Shiozawa M, Akaike M, Wada N, Rino Y, Masuda M, Tanaka K, Imada T. Clinicopathological significance of the gene expression of matrix metalloproteinases and reversion-inducing cysteine-rich protein with Kazal motifs in patients with colorectal cancer: MMP-2 gene expression is a useful predictor of liver metastasis from colorectal cancer. *Oncol*

Rep 2008; **19**: 1285-1291
35 **Gazitt Y**. Homing and mobilization of hematopoietic stem cells and hematopoietic cancer cells are mirror image processes, utilizing similar signaling pathways and occurring concurrently: circulating cancer cells constitute an ideal target for concurrent treatment with chemotherapy and antilineage-specific antibodies. *Leukemia* 2004; **18**: 1-10
36 **Liu B**, Yu J, Taylor L, Zhou X, Polgar P. Microarray and phosphokinase screenings leading to studies on ERK and JNK regulation of connective tissue growth factor expression by angiotensin II 1a and bradykinin B2 receptors in Rat1 fibroblasts. *J Cell Biochem* 2006; **97**: 1104-1120
37 **Li T**, Ling YH, Perez-Soler R. Tumor dependence on the EGFR signaling pathway expressed by the p-EGFR:p-AKT ratio predicts erlotinib sensitivity in human non-small cell lung cancer (NSCLC) cells expressing wild-type EGFR gene. *J Thorac Oncol* 2008; **3**: 643-647
38 **Pang H**, Rowan BG, Al-Dhaheri M, Faber LE. Epidermal growth factor suppresses induction by progestin of the adhesion protein desmoplakin in T47D breast cancer cells. *Breast Cancer Res* 2004; **6**: R239-R245
39 **Robinson DR**, Zylstra CR, Williams BO. Wnt signaling and prostate cancer. *Curr Drug Targets* 2008; **9**: 571-580
40 **Tanaka N**, Odajima T, Ogi K, Ikeda T, Satoh M. Expression of E-cadherin, alpha-catenin, and beta-catenin in the process of lymph node metastasis in oral squamous cell carcinoma. *Br J Cancer* 2003; **89**: 557-563
41 **Tachibana M**, Tonomoto Y, Hyakudomi R, Hyakudomi M, Hattori S, Ueda S, Kinugasa S, Yoshimura H. Expression and prognostic significance of EFNB2 and EphB4 genes in patients with oesophageal squamous cell carcinoma. *Dig Liver Dis* 2007; **39**: 725-732
42 **Nakamoto M**, Bergemann AD. Diverse roles for the Eph family of receptor tyrosine kinases in carcinogenesis. *Microsc Res Tech* 2002; **59**: 58-67
43 **Protzel C**, Kakies C, Kleist B, Poetsch M, Giebel J. Down-regulation of the metastasis suppressor protein KAI1/CD82 correlates with occurrence of metastasis, prognosis and presence of HPV DNA in human penile squamous cell carcinoma. *Virchows Arch* 2008; **452**: 369-375
44 **Yu J**, Becka S, Zhang P, Zhang X, Brady-Kalnay SM, Wang Z. Tumor-derived extracellular mutations of PTPRT / PTPrho are defective in cell adhesion. *Mol Cancer Res* 2008; **6**: 1106-1113
45 **Julien SG**, Dube N, Read M, Penney J, Paquet M, Han Y, Kennedy BP, Muller WJ, Tremblay ML. Protein tyrosine phosphatase 1B deficiency or inhibition delays ErbB2-induced mammary tumorigenesis and protects from lung metastasis. *Nat Genet* 2007; **39**: 338-346
46 **Sampaio C**, Dance M, Montagner A, Edouard T, Malet N, Perret B, Yart A, Salles JP, Raynal P. Signal strength dictates phosphoinositide 3-kinase contribution to Ras/extracellular signal-regulated kinase 1 and 2 activation via differential Gab1/Shp2 recruitment: consequences for resistance to epidermal growth factor receptor inhibition. *Mol Cell Biol* 2008; **28**: 587-600
47 **Ziegler DS**, Kung AL, Kieran MW. Anti-apoptosis mechanisms in malignant gliomas. *J Clin Oncol* 2008; **26**: 493-500
48 **Kerbauy DB**, Deeg HJ. Apoptosis and antiapoptotic mechanisms in the progression of myelodysplastic syndrome. *Exp Hematol* 2007; **35**: 1739-1746

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BRIEF ARTICLES

Prophylactic effect of glyceryl trinitrate on post-endoscopic retrograde cholangiopancreatography pancreatitis: A randomized placebo-controlled trial

Jian-Yu Hao, Dong-Fang Wu, Yue-Zeng Wang, Ying-Xin Gao, Hai-Po Lang, Wei-Zhen Zhou

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Abstract

AIM: To examine the prophylactic effect of glyceryl trinitrate on post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis and hyperamylasemia.

METHODS: Patients scheduled for ERCP were randomly divided into study group and placebo group. Patients in study group and placebo group were treated with 5 mg glyceryl trinitrate and 100 mg vitamin C, respectively, 5 min before endoscopic maneuvers.

RESULTS: A total of 74 patients were enrolled in the final analysis. Post-ERCP pancreatitis occurred in 3 patients (7.9%) of the study group and 9 patients (25%) in the placebo group (P = 0.012). Hyperamylasemia occurred in 8 patients of the study group (21.1%) and 13 patients (36.1%) of the placebo group (P = 0.037).

CONCLUSION: Glyceryl trinitrate before ERCP can effectively prevent post-ERCP and hyperamylasemia.

Key words: Glyceryl trinitrate; Cholangiopancreatography; Endoscopic retrograde; Pancreatitis

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is a widely applied method in the diagnosis and treatment of pancreatobiliary disease. Post-ERCP pancreatitis is the most common postoperative complication of ERCP. Although most cases of post-ERCP pancreatitis are mild, some may be severe and lethal. The incidence of post-ERCP pancreatitis is 1%-40%[1-3] and how to prevent it becomes an urgent clinical challenge. Some studies on drugs for preventing post-ERCP pancreatitis are available[4,5], but their results remain debatable. Therefore, most endoscopy centers do not give patients a conventional preventive drug therapy. Glyceryl trinitrate, a strong smooth muscle relaxant, is widely used in treatment of cardiovascular diseases. Glyceryl trinitrate could lower the basal pressure in the sphincter of Oddi and depress the resistance of bile outflow. Moretó *et al*[6] demonstrated that glycery trinitrate can reduce the incidence of post-ERCP pancreatitis. This prospective placebo-controlled double-blind randomized trial enrolled 74 patients scheduled for ERCP and observed the preventive effect of glycery trinitrate on post-ERCP pancreatitis.

MATERIALS AND METHODS

Study population

Seventy-four eligible patients at the age of 18 years and over were included in this study. ERCP was performed for them by the same experienced endoscopist.

Patients with acute or active chronic pancreatitis,

and a nitrate allergic history, and those undergone sphincterotomy, were excluded.

Research regimen

All the enrolled patients were randomly divided into study group and placebo group. Patients in study group took 5 mg sublingual glyceryl trinitrate 5 min before the procedure, while patients in placebo group took 100 mg sublingual vitamin C. Patients could receive antibiotics, analgesics or ataractics as needed, but somatostatin or octreotide was forbidden. Patients, operators or result observers were blinded to their grouping.

Observing targets

Serum amylase concentration in each patient was measured before and 4 and 24 h after endoscopy. Abdominal pain, fever, vomiting or other symptoms or signs were observed, and their laboratory or specifically evaluated results were recorded. Meanwhile, details of therapeutic endoscopic procedure, including expansion of bile duct, operating time (hours) and treatment, were also recorded.

Diagnostic criteria

According to the postoperative complications of ERCP[7,8], post-ERCP pancreatitis could be defined as a disease with sustained pancreatitis symptoms (such as abdominal pain) and high-amylase value over the normal value after ERCP. Hyperlipidemia was defined as the higher serum amylase concentration without or only with mild abdominal pain.

Statistical analysis

Data were analyzed using SPSS11.5 for statistics. Statistical analysis was performed by Student's *t*-test and χ^2-test.

RESULTS

General results

A total of 74 patients were randomly divided into study group (n = 36) and placebo group (n = 36). Of these patients, 6 were eliminated because of intubation failure, 1 had a BillrothII gastroectomy history, 2 did not allow endoscopy because of obstruction at duodenal descending part, and 3 failed to intubate the papilla. All the patients completed the trial. No significant difference was found in baseline characteristics between the two groups, such as gender, age, etiology, duct expansion, ERCP operating time, or treatment (Table 1).

Incidence of pancreatitis after ERCP

Post-ERCP pancreatitis occurred in 3 patients of the study group (7.9%), in 9 patients of the placebo group (25%), showing a significant difference between the two groups (P = 0.012). The condition of patients who developed post-ERCP pancreatitis was significantly improved after conservative treatment (Table 2).

Table 1 Baseline characteristics of study and placebo groups

	Study group	Placebo group	*P*
Demographic characteristics			
Number	38	36	
Sex ratio (M/F)	15/23	16/20	0.665
Mean age (yr)	64.29 ± 13.40	63.36 ± 15.13	0.781
Etiology			0.972
Choledocholithiasis (cases)	33	31	
Others(cases)	5	5	
Cholangiectasis (cases)	26	20	0.254
Treatment			0.841
Choledochostomy	26	26	
Stent intervention	6	6	
Sphincterotomy and drainage	6	4	
ERCP operating time (min)	36.89 ± 20.51	40.00 ± 24.73	0.558

Table 2 Complications occurred in study and placebo groups

Group	PEP	Hyperamylasemia	Normal
Study	3	8	27
Placebo	9	13	14
P	0.012	0.037	

Incidence of hyperamylasemia after ERCP

Hyperamylasemia occurred in 13 patients of the placebo group (36.1%) and 8 patients of the study group (21.1%). There was a significant difference between the two groups (P = 0.037, Table 2).

DISCUSSION

ERCP is an indispensable method for diagnosis and treatment of hepatic and pancreatobiliary disease. Pancreatitis is the most common postoperative complication of it. The nosogenesis may include[9]: (1) papilla edema due to reiterative intubation at duodenal papilla leading to pancreatic outflow obstruction, (2) pancreatic secretion caused by contrast agent over filling pancreatic duct or excessive contrast agent or bubbles entering the pancreas, (3) mechanical injury of pancreatic ducts and acini, (4) bacteria brought by imaging equipment or liquid infection in pancreatic duct or triggering original inflammation, (5) edema around pancreatic duct openings due to excessive coagulation in duodenal EST (EST) and impeding outflow of pancreatic secretion. Theoretically, post-ERCP pancreatitis could be reduced by mitigating papilla edema, keeping pancreatic and bile ducts open, controlling pancreatic secretion, avoiding contact of pancreatic tissue with active enzymes. Glyceryl trinitrate can relax smooth muscles not only in vascular wall but also in gastrointestinal tract, especially in the sphincter of Oddi. Sublingual glyceryl trinitrate shows its effect in 1-2 min and maintains its effect for 30 min. It also relaxes the sphincter of pancreatic and bile ducts when ERCP is performed, thus helping intubation and reducing spasm of sphincter of Oddi, keeping ducts open for contrast agent and pancreatin drainage, and reducing post-ERCP pancreatitis.

Sudhindran *et al*[10] suggested that sublingual glyceryl trinitrate (2 mg) before ERCP could relax sphincters, induce intubation and reduce 10% postoperative pancreatitis. Our study revealed that sublingual glyceryl trinitrate (5 mg) before ERCP could reduce pancreatitis and hyperamylasemia. Kaffes *et al*[11] showed that ransdermal GTN could not improve the success rate of ERCP cannulation or prevent post-ERCP pancreatitis in either average or high-risk patient groups.

There was a significant difference between the study and placebo groups. Compared with other drugs, glyceryl trinitrate is inexpensive, convenient and has less side-effects, and can be used as a prospective drug for preventing post-ERCP pancreatitis.

COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP) is a widely applied method for the diagnosis and treatment of pancreatobiliary disease. Post-ERCP pancreatitis is the most common postoperative complication of ERCP and how to prevent it has become an urgent clinical challenge.

Research frontiers

ERCP is an indispensable method for the diagnosis and treatment of hepatic and pancreatobiliary disease, and pancreatitis is the most common postoperative complication of it. There are some studies on drugs for preventing post-ERCP pancreatitis, but their results remain debatable. Therefore, most endoscopy centers do not give patients a conventional preventive drug therapy.

Innovations and breakthroughs

This trail revealed that sublingual glyceryl trinitrate (5 mg) before ERCP could reduce pancreatitis and hyperamylasemia.

Applications

Sublingual glyceryl trinitrate (5 mg) 5 min before the ERCP can prevent post-ERCP pancreatitis. Compared with other drugs, glyceryl trinitrate is inexpensive, convenient and has less side-effects, and can be as a prospective drug for preventing post-ERCP pancreatitis.

Terminology

Post-ERCP pancreatitis stands for post-endoscopic retrograde cholangio-pancreatography pancreatitis; ERCP stands for endoscopic retrograde cholangi opancreatography.

Peer review

Pancreatitis is the most common postoperative complication of ERCP. This study showed that glyceryl trinitrate could relax the sphincter of pancreatic and bile ducts when ERCP was performed, thus helping intubation and reducing the spasm of sphincter of Oddi, keeping ducts open for contrast agent and pancreatin drainage, and reducing post-ERCP pancreatitis. Glyceryl trinitrate is inexpensive, convenient and has less side-effect, and can be used a prospective drug for preventing post-ERCP pancreatitis.

REFERENCES

1 **Freeman ML**, Nelson DB, Sherman S, Haber GB, Herman ME, Dorsher PJ, Moore JP, Fennerty MB, Ryan ME, Shaw MJ, Lande JD, Pheley AM. Complications of endoscopic biliary sphincterotomy. *N Engl J Med* 1996; **335**: 909-918

2 **Sherman S**, Ruffolo TA, Hawes RH, Lehman GA. Complications of endoscopic sphincterotomy. A prospective series with emphasis on the increased risk associated with sphincter of Oddi dysfunction and nondilated bile ducts. *Gastroenterology* 1991; **101**: 1068-1075

3 **Sun FQ**, Zou DW, Li ZS, Xu GM, Sun ZX. Prevention of ERCP from pancreatitis. *Zhonghua Xiaohua Neijing Zazhi* 2000; **17**: 81-83

4 **Andriulli A**, Caruso N, Quitadamo M, Forlano R, Leandro G, Spirito F, De Maio G. Antisecretory vs. antiproteasic drugs in the prevention of post-ERCP pancreatitis: the evidence-based medicine derived from a meta-analysis study. *JOP* 2003; **4**: 41-48

5 **Andriulli A**, Clemente R, Solmi L, Terruzzi V, Suriani R, Sigillito A, Leandro G, Leo P, De Maio G, Perri F. Gabexate or somatostatin administration before ERCP in patients at high risk for post-ERCP pancreatitis: a multicenter, placebo-controlled, randomized clinical trial. *Gastrointest Endosc* 2002; **56**: 488-495

6 **Moretó M**, Zaballa M, Casado I, Merino O, Rueda M, Ramírez K, Urcelay R, Baranda A. Transdermal glyceryl trinitrate for prevention of post-ERCP pancreatitis: A randomized double-blind trial. *Gastrointest Endosc* 2003; **57**: 1-7

7 **Cotton PB**, Lehman G, Vennes J, Geenen JE, Russell RC, Meyers WC, Liguory C, Nickl N. Endoscopic sphincterotomy complications and their management: an attempt at consensus. *Gastrointest Endosc* 1991; **37**: 383-393

8 **Testoni PA**, Bagnolo F. Pain at 24 hours associated with amylase levels greater than 5 times the upper normal limit as the most reliable indicator of post-ERCP pancreatitis. *Gastrointest Endosc* 2001; **53**: 33-39

9 **Gottlieb K**, Sherman S. ERCP and biliary endoscopic sphincterotomy-induced pancreatitis. *Gastrointest Endosc Clin N Am* 1998; **8**: 87-114

10 **Sudhindran S**, Bromwich E, Edwards PR. Prospective randomized double-blind placebo-controlled trial of glyceryl trinitrate in endoscopic retrograde cholangio-pancreatography-induced pancreatitis. *Br J Surg* 2001; **88**: 1178-1182

11 **Kaffes AJ**, Bourke MJ, Ding S, Alrubaie A, Kwan V, Williams SJ. A prospective, randomized, placebo-controlled trial of transdermal glyceryl trinitrate in ERCP: effects on technical success and post-ERCP pancreatitis. *Gastrointest Endosc* 2006; **64**: 351-357

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Impact of *p27mt* gene on transplantation model of human colorectal cancer in nude mice

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Abstract

AIM: To investigate the inhibitory and anti-metastatic effect of mutant p27 gene (*p27mt*) on the growth of colorectal cancer xenografts in nude mice and its underlying mechanism.

METHODS: Inhibitory effect of *p27mt* gene on the growth of colorectal cancer xenografts was determined by measurement of tumor size before and after direct intra-tumoral injection of Ad-p27mt in a pre-established transplantation model of human colorectal cancer in nude mice. Cell cycle and apoptosis were detected by flow cytometry performed on single-cell suspension from an isolated tumor. Expression of MMP-9 in tumor tissue was detected by immunohistochemistry.

RESULTS: The average sizes of transplantation tumors were 1.94 ± 0.67 cm^3, 2.75 ± 0.83 cm^3 and 3.01 ± 0.76 cm^3 in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.05$). The average proliferation rates were 37.34% ± 1.45%, 53.16% ± 3.27% and 54.48% ± 2.43%, in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.05$). The average apoptosis rates were 19.79% ± 3.32%, 6.38% ± 4.91% and 7.25% ± 5.20% in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.01$). The average MMP-9 expression rates were 20%, 75% and 66.7% in the Ad-p27mt, Ad-LacZ and control groups, respectively ($P < 0.01$).

CONCLUSION: *p27mt* inhibits the growth of transplanted tumor by blocking the proliferation of cancer xenografts and by promoting apoptosis of transplantated tumor cells, as well as decrease transplanted tumor metastasis.

Key words: Colorectal cancer; *p27mt* gene; Nude mice; MMP-9

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INTRODUCTION

Along with the improvement of people's living standard and change in diet, there has been a gradual increase in the incidence of colorectal cancer[1]. None of the current treatment modalities for colorectal cancer, including surgery, radiotherapy and chemotherapy, is effective. With the advent of post-genomic era, the function of genes has become a priority research area and brought the dawn in gene therapy for tumor. Since *p27* is an anti-oncogene, this study was to evaluate the inhibitory and anti-metastatic effect of *p27mt* gene on the growth of colorectal cancer xenografts in nude mice and its underlying mechanism and to provide the theoretical basis for the use of *p27* in clinical treatment of colorectal cancer.

MATERIALS AND METHODS

Cell line and adenovirus

Lovo cell line, purchased from the Center for Type Culture Collection of Wuhan University, was cultured in RPMI 1640 medium. Working density of Levo cells

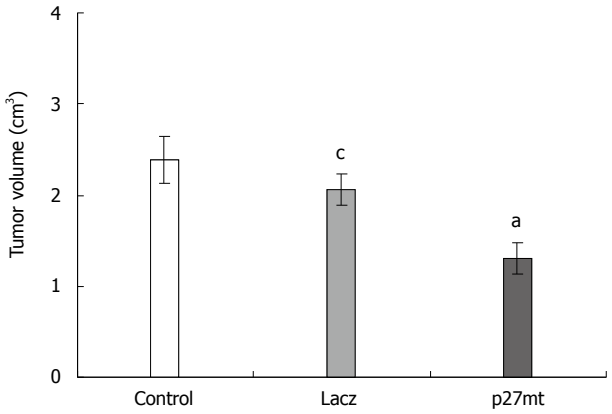

Figure 1 Comparison of transplanted tumor volume among different groups (cm^3). $^aP < 0.050$ *vs* Ad-Lacz, $^cP > 0.05$ *vs* control.

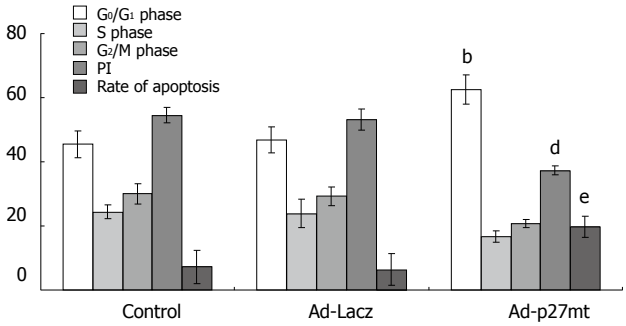

Figure 2 Comparison of status of cell cycle, PI and rate of apoptosis between different groups. $^bP < 0.01$ *vs* Ad-LacZ, $^dP < 0.01$ *vs* Ad-LacZ, $^eP < 0.01$ *vs* Ad-LacZ.

was 1×10^8/mL, with living cell count by trypan blue > 99%. Ad-LacZ was constructed and presented by Wang *et al*[2]. Ad-p27mt was self-constructed[3].

Establishment of transplantation model of colorectal cancer in nude mice and grouping

Thirty-six BALB/C nude mice, 4-6 wk old, and weighing 18-25 g, were purchased from the Laboratory Animal Management Center of Hubei Province. Lovo cell suspension (0.2 mL) was inoculated subcutaneously at the right back skin of each nude mouse. Upon tumor development, 27 nude mice whose tumor size was 0.5-1.5 cm in diameter were randomly assigned to control group, Ad-LacZ group or Ad-p27mt group. PBS (0.1 mL), Ad-LacZ (0.1 mL) with a virus density of 10^{10} pfu/mL, or Ad-p27mt (0.1 mL) with a virus density of 10^{10} pfu/mL was directly injected into the tumor of nude mice in the three groups, respectively, once every 3 d, for 28 d.

Determination of transplanted tumor size

Transplanted tumor size was calculated according to the following formula: $V = ab^2/2$, where a and b represent the length and width of the xenograft, respectively.

Flow cytometry

The 28th day after virus injection into the transplanted tumor, all mice were sacrificed with their tumors removed, weighed and photographed. Tumor tissue (15 g) was used in preparation of single cell suspension. Two hundred μL DNA-PREP™ LPR was mixed with 100 μL single cell suspension, and 1000 μL DNA-PREP stain was added into the mixture 3 min after the mixture was set at room temperature and protected from light. Fifteen min later, cell cycle and apoptosis were determined with a Coulter Epics XL flow cytometer. Proliferation index (PI) was calculated according to the following formula[4]:

$$PI = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$$

Immunohistochemical detection of MMP-9 expression

Anti-human mouse MMP-9 monoclonal antibody, S-P staining kit and DAB developer were obtained from Beijing Zhongshan Biotechnology, Co, Ltd. Since MMP-9 appears to be brown granules in cytoplasm, total cell number and the number of MMP-9 positive cells were counted in 5 visual fields of the matrix area around the tumor nest under microscope. Based on the scope and extent of staining, immunohistochemical results were logged according to the following criteria: "-" - no positively stained cells; "+" - cells lightly stained or < 10% cells stained; "++" - moderately stained or 10%-25% cells stained; "+++" - darkly stained or more than 50% cells stained, where - represents negative expression, +/++ stands for weakly positive expression, and + + + stands for strong expression.

Statistical analysis

One way-ANOVA was used in processing measurement data, which were expressed as mean ± SD. χ^2 test was adopted in calculation of enumeration data.

RESULTS

Comparison of transplanted tumor size between different groups

The average size of transplanted tumor in the Ad-p27mt group (1.94 ± 0.67 cm^3) was significantly smaller than that in the control group (3.01 ± 0.76 cm^3) ($P < 0.05$), no statistical significance was found in the average size of transplanted tumor between the two groups (3.01 ± 0.76 cm^3 *vs* 2.75 ± 0.83 cm^3) (Figure 1).

Comparison of cell cycle, PI and apoptosis between different groups

More cells at G_0/G_1 phase and less cells at S and G_2/M phase were observed in the Ad-p27mt group than the other two groups ($P < 0.05$). However, the difference between the two groups was insignificant ($P < 0.05$, Figure 2).

MMP-9 expression in transplanted tumor

Since MMP-9 is mainly found in the matrix area around tumor nest, brown stained granules were observed in cytoplasm of cancer cells (Figure 3). MMP-9 expression rate for the Ad-p27mt group was significantly decreased compared with control group (Table 1).

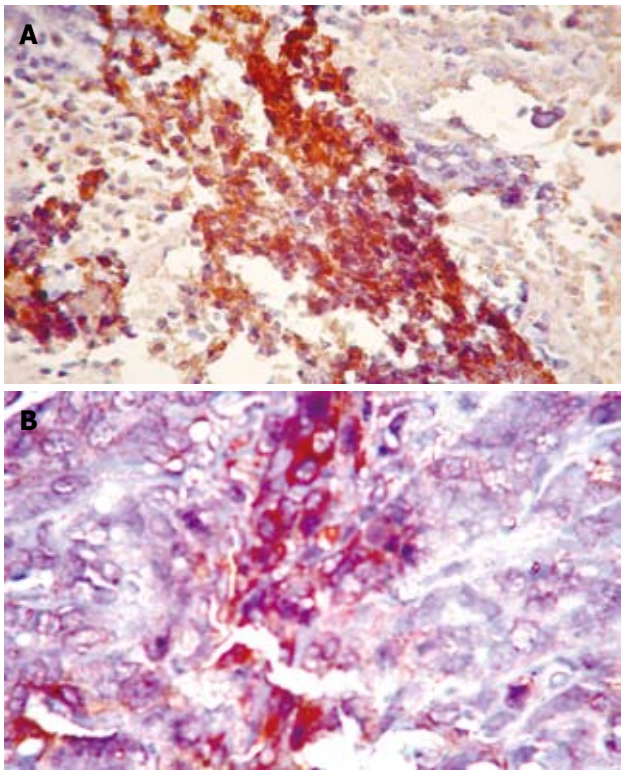

Figure 3 Expression of MMP-9 in the transplanted tumor. A: control group; B: Ad-p27mt group.

DISCUSSION

While *p27* is a negative regulator of cell cycle and a tumor suppressor[5], tumor may develop when abnormal (missing or decreased) expression of *p27* and attenuated inhibition on cell cycle lead to uncontrolled cell growth and carcinogenesis[6]. The results of studies demonstrate that decreased *p27* expression was associated with ubiquitin-mediated proteasome phosphorylation and abnormal activity of *p27*[7,8]. We investigated the *in vivo* inhibitory effect of *p27* on transplanted tumor by intratumoral injection of mutated *p27mt* adenovirus.

Park *et al*[9] found that inhibition of mutant *p27* (*p27mt*) on tumor cells seem stronger than that of wild type *p27* (*p27wt*) as demonstrated in cells arrested in G_0/G_1 phase, and that the apoptosis promoting activity of *p27mt* is also stronger. Another study revealed that the half-life of *p27mt* is over 12 h, much longer than that of *p27wt* (2 h)[10]. Through determination of the size of transplanted tumor, this study displayed that *p27mt* gene significantly inhibited the growth of colorectal cancer by inhibiting cell proliferation and by promoting cell apoptosis, suggesting that *p27mt* can evidently suppress cell proliferation at G_0/G_1 phase. The apoptosis promoting activity of *p27mt* was more obvious in control group, while the apoptosis rate of *p27wt* was up to 37.9% ± 3.32%.

It was reported in our preliminary study that the expression level of *p27* in colorectal cancer tissue is quite low[11]. In this study, the expression of MMP-9 was significantly decreased in *p27mt* group. MMP-9, in the form of proenzyme in cytoplasm, when released under physiological condition, may degrade extracellular matrix and is involved in development of human body and multiple physiological processes including tissue repair[12]. When disturbance of MMP-9 gene occurs, increased proenzyme leads to escalated degradation of extracellular components, including Ⅳ and Ⅴ collagen and laminin, and undermined integrity of basement membrane. Therefore, MMP-9 plays a very important role in the process of tumor metastasis[13]. In this study, a reduced MMP-9 expression was observed after *p27mt* was injected. No tumor metastasis was found with in 28 d after transplantation of the tumor.

In conclusion, *p27mt* inhibits the growth of colorectal cancer by inhibiting cancer cell proliferation and promoting cell apoptosis as well as metastasis of colorectal cancer.

Table 1 Comparison of MMP-9 positive rate between different groups

Group	-	+	++	+++	Positive rate (%)
Control	12	4	11	9	66.7
Ad-LacZ	9	2	14	11	75[a]
Ad-p27mt	30	2	4	0	20[c]

[a]$P > 0.05$ *vs* control group; [c]$P < 0.05$ *vs* Ad-LacZ group and control group.

COMMENTS

Background

Along with the improvement in people's living standard and changes in diet, there has been a gradual increase in the incidence of colorectal cancer in China. However, no effective therapeutic modalities are available for it. Gene therapy for restoration of p27 expression is a promising therapy for it. A mutant type of p27 gene, with mutant of Thr-187/Pro-188 to Met-187/Ile-188, can inhibit degradation of *p27* protein through the ubiquitin-mediated pathway. The inhibitory effect of mutant *p27 (p27mt)* seems stronger than that of wild type *p27 (p27wt)* on tumor cells, as demonstrated by cells arrested in the G_0/G_1 phase. The apoptosis promoting activity of *p27mt* is also stronger. However, no study about its effect on colorectal cancer is available.

Research frontiers

p27, a cyclin-dependent kinases inhibitor, a tumor suppressor gene, and a promoter of apoptosis, has been widely investigated. Anti-tumor activity of *p27* has been demonstrated in breast, lung, and oral cancer. However, the anti-tumor bioactivity of *p27mt* has not been studied on colorectal cancer.

Innovations and breakthroughs

The results of this study indicate that *p27mt* gene has a strong anti-tumor bioactivity on colorectal cancer *in vivo* and *in vitro*.

Applications

This gene may be developed into a new therapeutic agent for colorectal cancer.

Peer review

This study showed the effect of over-expression of a mutant form of *p27* on colorectal cancer growth in a xenotransplantation model. The results are largely descriptive and the effect of *p27mt* seems modest on tumor growth. Further study is needed to show the expression of transfected *p27mt* gene.

REFERENCES

1 **Zheng S**, Cai SR. Colorectal Cancer Epidemiology and Prevention Study in China. *Chin Ger J Clin Oncol* 2003; **2**: 72-75

2 **Wang JN**, Huang YZ, Kong X, Guo LY. The construction of recombinant adenoviral plasmid by homologous recombination in bacteria and the preparation of recombinant

adenovirus expressing β-galactosidase. *Yunyang Yixueyuan Xuebao* 2004; **23**: 1-5

3 **Chen J**, Xu SY, Deng CS, Wang JN, Huang YZ. Efficient generation of human mutant p27 recombinant adenovirus by homologous recombination in bacteria. *J Fourth Mil Med Univ* 2004; **5**: 406-409

4 **Cai FG**, Xiao JS, Ye QF. Effects of ischemic preconditioning on cyclinD1 expression during early ischemic reperfusion in rats. *World J Gastroenterol* 2006; **12**: 2936-2940

5 **Nan KJ**, Jing Z, Gong L. Expression and altered subcellular localization of the cyclin-dependent kinase inhibitor p27Kip1 in hepatocellular carcinoma. *World J Gastroenterol* 2004; **10**: 1425-1430

6 **Fukunaga M**. Immunohistochemical characterization of cyclin E and p27KIP1 expression in early hydatidiform moles. *Int J Gynecol Pathol* 2004; **23**: 259-264

7 **Ganoth D**, Bornstein G, Ko TK, Larsen B, Tyers M, Pagano M, Hershko A. The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. *Nat Cell Biol* 2001; **3**: 321-324

8 **Troncone G**, Martinez JC, Iaccarino A, Zeppa P, Caleo A, Russo M, Migliaccio I, Motti ML, Califano D, Palmieri EA, Palombini L. p27Kip1 is expressed in proliferating cells in its form phosphorylated on threonine 187. *BMC Clin Pathol* 2005; **5**: 3

9 **Park KH**, Seol JY, Kim TY, Yoo CG, Kim YW, Han SK, Shim YS, Lee CT. An adenovirus expressing mutant p27 showed more potent antitumor effects than adenovirus-p27 wild type. *Cancer Res* 2001; **61**: 6163-6169

10 **Hurteau JA**, Brutkiewicz SA, Wang Q, Allison BM, Goebl MG, Harrington MA. Overexpression of a stabilized mutant form of the cyclin-dependent kinase inhibitor p27(Kip1) inhibits cell growth. *Gynecol Oncol* 2002; **86**: 19-23

11 **Sun ZQ**, Xu SY, Deng CS, Zhang L,Tian L. Expression of p38 Protein and Its Clinical Significance in Human Colorectal Carcinomas. *Yunyang Yixueyuan Xuebao* 2005; **24**: 151-153

12 **Zhang YF**, Wang ZR, Niu XJ, Wang L, Li HL. Expression of MMP-9 and CD44v6 in gallbladder carcinoma and implication. *Zhonghua Shiyan Waike Zazhi* 2002; **19**: 405-406

13 **Wan YL**, Rong L, Liu YS, Wang X, Wu T, Pan YS, Yao HW, Ye JM, Tang JQ, Zhou J. The role of tissue factor in the invasion of colorectal carcinoma and its relationship with matrix metalloproteinase-9. *Zhonghua Shiyan Waike Zazhi* 2004; **21**: 1087-1088

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CASE REPORT

Pedunculated Brunner's gland hamartoma of the duodenum causing upper gastrointestinal hemorrhage

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Abstract

A case of pedunculated Brunner's gland hamartoma (BGH) of the duodenum causing upper gastrointestinal (GI) hemorrhage is reported. The patient was a 47-year-old man who visited our hospital for further evaluation of tarry stools and shortness of breath. Endoscopic examination of the upper digestive tract revealed a large peduncular polyp with bleeding, about 30 mm in diameter, arising from the wall of the second portion of the duodenum. GI bleeding occurred from the base of the stalk of the polyp. Endoscopic polypectomy was performed. Histological examination of the specimen revealed that the main body of the polyp contained several lobules of mature Brunner's gland with areas of cystic dilatation. The surface epithelium consisted of normal duodenal mucosa with areas of focal ulceration. This polyp was diagnosed as a BGH. The symptom of tarry stools resolved after endoscopic resection. Our case shows that treatment is necessary for duodenal BGH if GI bleeding occurs.

Key words: Brunner's gland; Hyperplasia; Duodenal polyp; Endoscopic polypectomy; Gastrointestinal bleeding

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Hirasaki S, Kubo M, Inoue A, Miyake Y, Oshiro H. Pedunculated Brunner's gland hamartoma of the duodenum causing upper gastrointestinal hemorrhage. *World J Gastroenterol* 2009; 15(3): 373-375 Available from: URL: http://www.wjgnet.com/1007-9327/15/373.asp DOI: http://dx.doi.org/10.3748/wjg.15.373

INTRODUCTION

Brunner's gland hyperplasia and hamartoma are infrequently encountered polypoid nodules and masses in the proximal duodenum[1]. Brunner's gland hamartoma (BGH) is a very rare cause of upper gastrointestinal (GI) hemorrhage. Clinically, patients may present with symptoms of duodenal obstruction or upper GI hemorrhage and require endoscopic or surgical excision[2]. Herein, we describe a relatively rare case of pedunculated BGH of the duodenum causing upper GI hemorrhage.

CASE REPORT

A 47-year-old man presented with the symptoms of tarry stools and shortness of breath. He was in good health with no specific family or past medical history. His body temperature was 36.5℃, blood pressure was 148/82 mmHg, and radial pulse rate was 70 beats/min and regular. He had anemia, but no jaundice. Neurological examination revealed no abnormal findings. Laboratory tests showed a red blood cell count of $318 \times 10^4/\mu L$ [normal range (NR), 430×10^4-$570 \times 10^4/\mu L$], a white blood cell count of $7600/\mu L$, a platelet count of $30.8 \times 10^4/\mu L$, and a hemoglobin concentration of 8.9 g/dL (NR, 14-18 g/dL). Endoscopic examination of the upper digestive tract revealed a large peduncular polyp, about 30 mm in diameter, arising from the wall of the second portion of the duodenum (Figure 1A). The polyp head was lobulated. GI bleeding occurred from the base of stalk of the polyp (Figure 1B and C). It was suspected to be a BGH, from the endoscopic findings. An air-contrast barium meal also revealed a pedunculated polyp in the second portion of the duodenum (Figure 2). There was no lesion in the esophagus and stomach. Endoscopic polypectomy was performed (Figure 3). The cut surface of the resected specimen showed an approximately 3-cm whitish mass (Figure 4). Histological examination of the specimen revealed that the main body of the polyp contained several lobules of mature Brunner's gland with areas of cystic dilatation (Figure 5). The surface

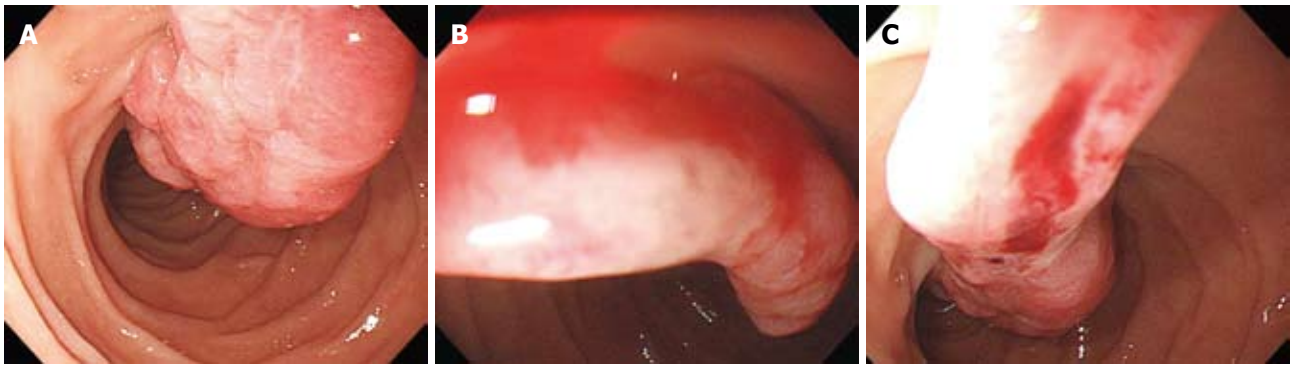

Figure 1 Endoscopy images. A large lobulated peduncular polyp, about 30 mm in diameter, in the second portion of the duodenum (A and B) and bleeding from the base of the stalk of the polyp (C).

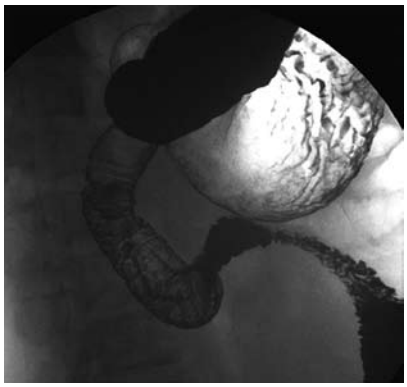

Figure 2 Double contrast radiograph of the duodenum showing an approximately 3-cm peduncular polyp.

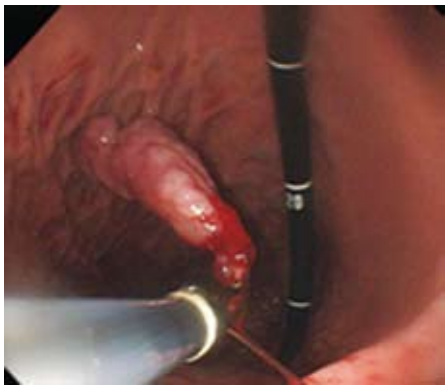

Figure 3 Endoscopic polypectomy was performed and the resected specimen was moved into the stomach.

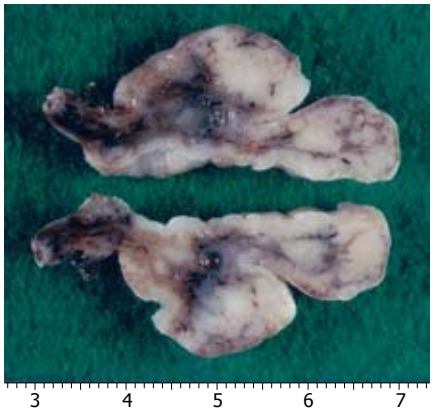

Figure 4 Macroscopic findings of the polypectomy specimen. The cut surface showed an approximately 3-cm whitish mass.

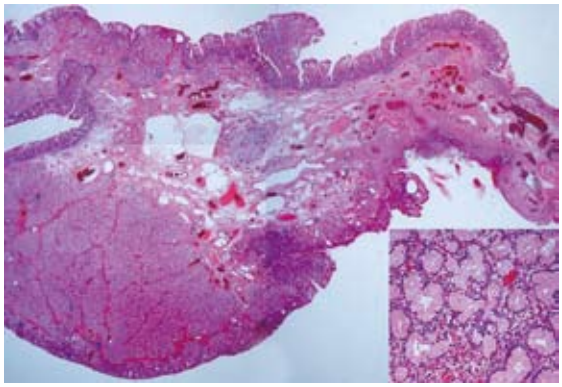

Figure 5 Microscopic findings of the polypectomy specimen. Low-power view of a cross section showing submucosal proliferation of Brunner's gland below the duodenal mucosa (HE x 10, x 100).

epithelium consisted of normal duodenal mucosa with areas of focal ulceration. The base of the stalk was ulcerated. There was no evidence of malignancy. The lesion was diagnosed as a pedunculated BGH of the duodenum. After endoscopic polypectomy, the symptom of tarry stools resolved.

DISCUSSION

The etiology of BGH remains obscure. BGH is present mostly in middle age without any gender predominance[3]; however, cases have been described from early infancy to even 80 years of age. BGHs have a broad range of sizes (0.5-12 cm)[1,4-6]. The most common location is the posterior wall of the duodenum near the junction of the first and second portions. BGHs were found in the duodenal bulb in 70% of cases, in the second portion of the duodenum in 26%, and in the third portion in 4% in one series of 27 patients by Levine *et al*[3]. In the present case, it was located in the second portion of the duodenum.

BGHs are usually asymptomatic and often detected

incidentally on barium meal or endoscopy[1,6]. Another review of the literature has revealed that the main clinical feature of BGH is GI bleeding or bowel obstruction[1,2,4,6,7]. The diagnosis of BGH is usually made by a combination of radiographic and endoscopic findings. The radiographic finding of large localized BGHs is a sessile or pedunculated polypoid filling defect[1,6,8]. Endoscopic characteristic findings, like radiographic findings, are as follows: (1) pedunculated polyp, although 11% can be sessile[3,9,10]; (2) polypoid or lobulated mass; and (3) covered with normal mucosa[3,11,12]. BGH should be distinguished from other duodenal lesions such as leiomyoma, polypoid adenoma of the superficial mucosal glands, aberrant pancreatic tissue and malignant tumors[1,4,13,14]. Diagnosis is rarely conclusive on endoscopic biopsies because the lesion locates mainly in the submucosal layer, and the biopsy is often not deep enough to reach the submucosal tumor tissue[1,6,13]. The final diagnosis of BGH depends on the pathological findings of resected specimens obtained by endoscopic mucosal resection, polypectomy or surgical treatment.

As to therapy, BGH of the duodenum can best be removed endoscopically, because it is thought to be clinically and histologically benign. However, endoscopists should be aware that there have been rare case reports of malignancy arising from Brunner's gland[1,4,15,16]. Endoscopic or surgical treatment is necessary if GI bleeding occurs[4,7,11-14], as in the present case. GI bleeding, typically manifested by hematemesis or melena, from ulceration or erosion of the mucosa stretched over the submucosal lesion, may occur and can occasionally be massive and rarely fatal[3,10,13]. In the present case, bleeding was caused by ulceration of the stalk. The mechanism underlying GI bleeding in the present case was atypical.

In conclusion, we report a case of pedunculated BGH of the duodenum causing upper GI hemorrhage. BGH should be generally taken into consideration as a differential diagnosis of duodenal masses. BGH is not fatal and patients remain asymptomatic in their daily lives, except for GI bleeding or bowel obstruction. Endoscopists should be aware that BGH may exhibit the aforementioned endoscopic characteristics and may cause GI bleeding.

REFERENCES

1 **Patel ND**, Levy AD, Mehrotra AK, Sobin LH. Brunner's gland hyperplasia and hamartoma: imaging features with clinicopathologic correlation. *AJR Am J Roentgenol* 2006; **187**: 715-722

2 **Matsumoto T**, Iida M, Matsui T, Yao T, Fujishima M. A large Brunner's gland adenoma removed by endoscopic polypectomy. *Endoscopy* 1990; **22**: 192-193

3 **Levine JA**, Burgart LJ, Batts KP, Wang KK. Brunner's gland hamartomas: clinical presentation and pathological features of 27 cases. *Am J Gastroenterol* 1995; **90**: 290-294

4 **Chattopadhyay P**, Kundu AK, Bhattacharyya S, Bandyopadhyay A. Diffuse nodular hyperplasia of Brunner's gland presenting as upper gastrointestinal haemorrhage. *Singapore Med J* 2008; **49**: 81-83

5 **Nakanishi T**, Takeuchi T, Hara K, Sugimoto A. A great Brunner's gland adenoma of the duodenal bulb. *Dig Dis Sci* 1984; **29**: 81-85

6 **Gao YP**, Zhu JS, Zheng WJ. Brunner's gland adenoma of duodenum: a case report and literature review. *World J Gastroenterol* 2004; **10**: 2616-2617

7 **de Nes LC**, Ouwehand F, Peters SH, Boom MJ. A large Brunner's gland hamartoma causing gastrointestinal bleeding and obstruction. *Dig Surg* 2007; **24**: 450-452

8 **Peetz ME**, Moseley HS. Brunner's gland hyperplasia. *Am Surg* 1989; **55**: 474-477

9 **Brookes MJ**, Manjunatha S, Allen CA, Cox M. Malignant potential in a Brunner's gland hamartoma. *Postgrad Med J* 2003; **79**: 416-417

10 **Zangara J**, Kushner H, Drachenberg C, Daly B, Flowers J, Fantry G. Iron deficiency anemia due to a Brunner's gland hamartoma. *J Clin Gastroenterol* 1998; **27**: 353-356

11 **Kehl O**, Buhler H, Stamm B, Amman RW. Endoscopic removal of a large, obstructing and bleeding duodenal Brunner's gland adenoma. *Endoscopy* 1985; **17**: 231-232

12 **Block KP**, Frick TJ, Warner TF. Gastrointestinal bleeding from a Brunner's gland hamartoma: characterization by endoscopy, computed tomography, and endoscopic ultrasound. *Am J Gastroenterol* 2000; **95**: 1581-1583

13 **Schluger LK**, Rotterdam H, Lebwohl O. Gastrointestinal hemorrhage from a Brunner's gland hamartoma. *Am J Gastroenterol* 1994; **89**: 2088-2089

14 **Walden DT**, Marcon NE. Endoscopic injection and polypectomy for bleeding Brunner's gland hamartoma: case report and expanded literature review. *Gastrointest Endosc* 1998; **47**: 403-407

15 **Christie AC**. Duodenal carcinoma with neoplastic transformation of the underlying Brunner's glands. *Br J Cancer* 1953; **7**: 65-67

16 **Fujimaki E**, Nakamura S, Sugai T, Takeda Y. Brunner's gland adenoma with a focus of p53-positive atypical glands. *J Gastroenterol* 2000; **35**: 155-158

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LETTERS TO THE EDITOR

Agenesis of the dorsal pancreas

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Abstract

During the last 100 years in medical literature, there are only 54 reports, including the report of Pasaoglu *et al* (*World J Gastroenterol* 2008; 14: 2915-2916), with clinical descriptions of agenesis of the dorsal pancreas in humans. Agenesis of the dorsal pancreas, a rare congenital pancreatic malformation, is associated with some other medical conditions such as hyperglycemia, abdominal pain, pancreatitis and a few other diseases. In approximately 50% of reported patients with this congenital malformation, hyperglycemia was demonstrated. Evaluation of hyperglycemia and diabetes mellitus in all patients with agenesis of the dorsal pancreas including description of fasting blood glucose, oral glucose tolerance test, glycated hemoglobin and medical treatment would be a future goal. Since autosomal dominant transmission has been suggested in single families, more family studies including imaging technologies with demonstration of the pancreatic duct system are needed for evaluation of this disease. With this letter to the editor, we aim to increase available information for the better understanding of this rare disease.

Key words: Agenesis of dorsal pancreas; Diabetes mellitus; Glucose intolerance; Abdominal pain; Pancreatitis

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Schnedl WJ, Piswanger-Soelkner C, Wallner SJ, Krause R, Lipp RW. Agenesis of the dorsal pancreas. *World J Gastroenterol* 2009; 15(3): 376-377 Available from: URL: http://www.wjgnet.com/1007-9327/15/376.asp DOI: http://dx.doi.org/10.3748/wjg.15.376

TO THE EDITOR

We read with interest the published case report of Pasaoglu *et al* on agenesis of the dorsal pancreas[1]. Agenesis of the dorsal pancreas is a rare congenital pancreatic malformation and may be associated with some other medical conditions and diseases. During the last 100 years in medical literature, we know of 54 reports with clinical descriptions of agenesis of the dorsal pancreas in humans. We recently summarized systematically all reported patients with agenesis of the dorsal pancreas and discussed the associated medical conditions and diseases[2]. In 1911, the first description of agenesis of the dorsal pancreas was published as an autopsy finding. Now, as new imaging technologies have been developed and improved, the number of patients reported to show agenesis of the dorsal pancreas has increased rapidly over the last years. So far, the findings in autopsy and the radiological descriptions of anatomic pancreatic structures are highly variable and in most cases there is a diagnosis made without description of the pancreatic duct system[2]. In some patients, an enlarged or prominent or compensatory hypertrophy of the pancreatic head is described, whereas other descriptions include normal sized pancreatic head, as well as mildly atrophic and small head of the pancreas. We support the description of Pasaoglu *et al*[1] and confirm that the diagnosis of agenesis of the dorsal pancreas is inconclusive without demonstration of the absence of the dorsal pancreatic duct, either with endoscopic retrograde or magnetic resonance pancreatography.

Diabetes mellitus comprises a group of metabolic diseases characterized by hyperglycemia resulting from

defects in insulin secretion and/or insulin action. Criterion for diagnosis of diabetes mellitus is a fasting blood glucose > 126 mg/dL. Another diagnostic test to differentiate impaired glucose tolerance and diabetes mellitus is the oral glucose tolerance test[3]. Single familial observations of agenesis of the dorsal pancreas suggest autosomal dominant transmission. In one family with agenesis of the dorsal pancreas, a marked defect in hepatic glycogen metabolism, even in non-diabetic offspring, is demonstrated. An impaired index of the first phase insulin secretion in the diabetic and in both non-diabetic family members is described. Since the root cause of most common diabetes mellitus, type 1 and type 2 diabetes, is a decrease in-cell mass, this can be related to reduced-cell mass and might contribute to the development of glucose intolerance which ultimately leads to diabetes mellitus[4].

We suggest that more family studies including imaging technologies with demonstration of the pancreatic duct system are needed. Evaluation of hyperglycemia in all patients with agenesis of the dorsal pancreas including description of fasting blood glucose, oral glucose tolerance test, glycated hemoglobin and medical treatment would be a future goal.

REFERENCES

1 **Pasaoglu L**, Vural M, Hatipoglu HG, Terekliogl u G, Koparal S. Agenesis of the dorsal pancreas. *World J Gastroenterol* 2008; **14**: 2915-2916

2 **Schnedl WJ**, Piswanger-Soelkner C, Wallner SJ, Reittner P, Krause R, Lipp RW, Hohmeier HE. Agenesis of the dorsal pancreas and associated diseases. *Dig Dis Sci* 2009; **54**: 481-487

3 Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2008; **31** Suppl 1: S55-S60

4 **Stingl H**, Schnedl WJ, Krssak M, Bernroider E, Bischof MG, Lahousen T, Pacini G, Roden M. Reduction of hepatic glycogen synthesis and breakdown in patients with agenesis of the dorsal pancreas. *J Clin Endocrinol Metab* 2002; **87**: 4678-4685

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LETTERS TO THE EDITOR

Another new variant of Bouveret's syndrome

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Abstract

Although Bouveret's syndrome, i.e. gastric outlet obstruction by a large gallstone impacted in the proximal duodenum secondary to a cholecystoduodenal fistula, is rare, its pathogenesis and clinical features are well characterized. However, existence of variant forms of the syndrome are not well known, and as far as we know, only two cases of variant Bouveret's syndrome have been described in the English-language literature. We present a case of another new variant of Bouveret's syndrome in a 54-year-old Korean woman.

Key words: Duodenal obstruction; Biliary fistula; Gallstones

Park SH, Lee SW, Song TJ. Another new variant of Bouveret's syndrome. *World J Gastroenterol* 2009; 15(3): 378-379 Available from: URL: http://www.wjgnet.com/1007-9327/15/378.asp DOI: http://dx.doi.org/10.3748/wjg.15.378

TO THE EDITOR

We read with much interest the case report of Arioli *et al*[1], 'a new variant of Bouveret's syndrome', recently published in *World Journal of Gastroenterology*. They described a case of gallstone ileus in which an incomplete pyloric occlusion developed following the migration of a large stone through a cholecystogastric fistula, in a patient with undiagnosed chronic cholecystitis. In their case, the gallstone lay in the gastric antrum rather than within the lumen of the duodenum, which is different from typical Bouveret's syndrome. Doody *et al*[2] have also described a variant of Bouveret's syndrome in which duodenal obstruction was caused by a huge gallstone that lay outside the duodenum, which gave an atypical Bouveret's syndrome appearance. We recently encountered a similarly interesting case of gastric outlet obstruction caused by complicated gallstone disease. A 54-year-old Korean woman was admitted to the hospital with epigastric pain and intermittent vomiting of 20 d duration. She had a history of recurrent bouts of right

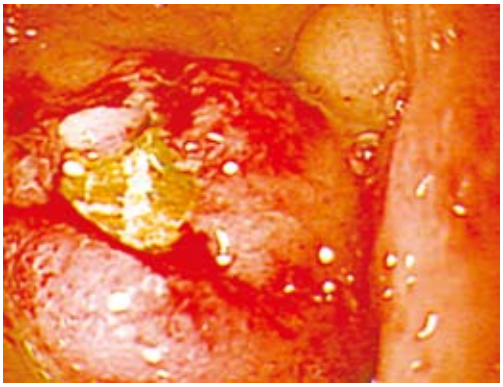

Figure 1 Gastroduodenal endoscopy revealed an ovoid mass in the first portion of the duodenum, the center of which harbored a mucosal defect.

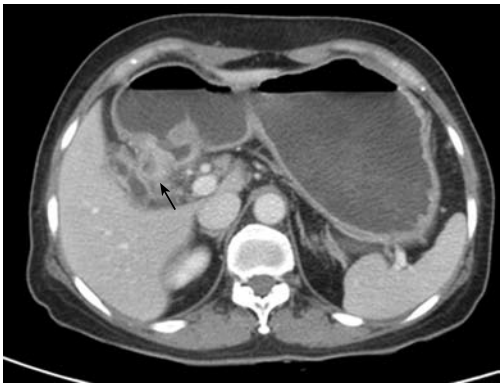

Figure 2 Abdominal CT showed wall thickening at the bulbar and postbulbar portions of the duodenum, with marked gastric dilatation. A cystic lesion (arrow) was found at the thickened duodenal wall. The gallbladder was partially collapsed and adhered to the duodenum.

upper quadrant abdominal pain caused by acute cholecystitis. Gastroduodenoscopy showed an obstructive duodenal mass, the center of which harbored a mucosal defect (Figure 1). Abdominal computed tomography (CT) showed a cystic lesion within the thickened wall of the bulbar and post-bulbar portions of the duodenum (Figure 2). The gallbladder contained a small stone and was partially collapsed. On laparotomy, the gallbladder was densely adhered to the first portion of the duodenum. The patient underwent cholecystectomy, with excision of a portion of the duodenal wall. In the cystic lesion of the duodenal wall, muddy stone fragments were found. Based on operative findings, it was certain that a large gallstone had passed through the cholecystoduodenal fistula into the duodenal wall, and remained there sufficiently long to form a submucosal mass that eroded through the mucosa into the duodenal lumen. Although the stone was passed without causing any further problems, the cavaitary lesion in the duodenal wall persisted and bulged into the duodenal lumen, which caused the duodenal obstruction. The features of our patient resembled a Bouveret's syndrome in clinical presentation, but differed from it inasmuch that the duodenal obstruction was not caused by a gallstone per se, but rather by secondary changes in the duodenal wall caused by a gallstone. A cystic lesion within the wall of the duodenum caused confusion in our case, and surgical exploration was required to make a definite diagnosis and to determine a treatment strategy. We think that this case may be another new variant of Bouveret's syndrome.

REFERENCES

1 **Arioli D**, Venturini I, Masetti M, Romagnoli E, Scarcelli A, Ballesini P, Borghi A, Barberini A, Spina V, De Santis M, Di Benedetto F, Gerunda GE, Zeneroli ML. Intermittent gastric outlet obstruction due to a gallstone migrated through a cholecysto-gastric fistula: a new variant of "Bouveret's syndrome". *World J Gastroenterol* 2008; **14**: 125-128

2 **Doody O**, Ward E, Buckley O, Hogan B, Torreggiani WC. Bouveret's syndrome variant. *Digestion* 2007; **75**: 126-127

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
www.asco.org/annualmeeting

May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
DDW 2009
http://www.ddw.org

June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists(IASGO)
http://iasgo2009.org/en/index.shtml

September 27-30, 2009
Taipei, China
Asian Pacific Digestive Week
http://www.apdwcongress.org/2009/index.shtml

October 7-11, 2009
Boston Park Plaza Hotel and Towers, Boston, MA, United States
Frontiers in Basic Cancer Research

October 13-16, 2009
Hyatt Regency Mission Bay Spa and Marina, San Diego, CA, United States
Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

October 30-November 3, 2009
Boston, MA, United States
The Liver Meeting

November 15-19, 2009
John B. Hynes Veterans Memorial Convention Center, Boston, MA, United States
AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

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Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462

PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Italics

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